THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Habib Zaghouani
Appl. No.:	10/681,788
Conf. No.:	6701
Filed:	October 8, 2003
Title:	SUSTAINED TREATMENT OF TYPE 1 DIABETES AFTER EXPRESSION OF PREDISPOSITION MARKERS
Art Unit:	1644
Examiner:	Edwoldt, G.R.
Docket No.:	3718027.00005

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANTS' APPEAL BRIEF

Sir:

Appellants submit this Appeal Brief in support of the Notice of Appeal filed on January 30, 2012. This Appeal is taken from the Final Office Action dated July 29, 2011.

I. REAL PARTIES IN INTEREST

The real parties in interest for the above-identified patent application on Appeal is The Curators of The University of Missouri by virtue of Assignments recorded on November 21. 2006 at reel/frame 018542/0168 in the United States Patent and Trademark Office.

II. RELATED APPEALS AND INTERFERENCES

Appellant's legal representative and the Assignee of this patent application submit that this application (U.S. 10/681,788) was previously appealed and that the Examiner re-opened prosecution of this application in view of the Appeal Brief filed on January 21, 2010. Appellant's legal representative and the Assignee of this patent application further submit that a Notice of Appeal and an Appeal Brief has been filed on May 10, 2011 and July 11, 2011, respectively, in a continuation-in-part application (U.S. 11/290,070) and that a Notice of Appeal and Appeal Brief has been filed on May 10, 2011 and July 11, 2011, respectively, in a continuation-in-part application (U.S. 11/425,084). Appellant's legal representative and the Assignee of this patent application further submit that a Reply Brief was filed on December 19, 2011 in U.S. 11/290,070 and U.S. 11/425,084. Appellant's legal representative and the Assignee of this patent application further submit that they do not know of any interferences or judicial proceedings that may be related to, directly affect or be directly affected by or have a bearing on the Board's decision with respect to the above-identified Appeal.

III. STATUS OF CLAIMS

Claims 1-5, 7-13 and 15-30 are pending in this application. Claims 6 and 14 were previously cancelled and claims 8-12, 20, 21 and 25 are withdrawn. Claims 1-5, 7, 13, 15-19, and 22-30 stand rejected. Therefore, claims 1-5, 7, 13, 15-19, and 22-30 (with claim 1 in independent form) are being appealed in this Brief. The appealed claims are reproduced in the Claims Appendix.

IV. STATUS OF AMENDMENTS

A Preliminary Amendment was filed on June 19, 2007. A first Non-Final Office Action was mailed on August 20, 2007. Appellants filed a Response to the first Non-Final Office Action on December 19, 2007. A Final Office Action was mailed on March 28, 2008. Appellants subsequently filed a request for continued examination on August 8, 2008. A Final Office Action was mailed on October 6, 2008. Appellants filed a Notice of Appeal on October 31, 2008 and an Appeal Brief on January 21, 2010 with respect to a Final Office Action dated October 6, 2008. The Examiner reopened prosecution in this case with the Non-Final Office Action dated April 8, 2010. Appellants filed a response on September 15, 2010. A Non-Final Office Action was mailed on November 22, 2010. Appellants filed a response on May 20, 2011. A Final Office Action was mailed on July 29, 2011 (attached hereto as EXHIBIT A in the Evidence Appendix). A Notice of Appeal was then filed on January 30, 2012.

V. SUMMARY OF CLAIMED SUBJECT MATTER

A summary of the claimed subject matter by way of reference to the specification and/or figures for sole independent claim 1 is provided as follows:

A method of preventing or delaying onset of Type 1 diabetes in a subject in need thereof (Page 4, lines 11-14), the method comprising administering to the subject a pharmaceutically acceptable composition comprising a soluble fusion protein (page 25, line 9), wherein the fusion protein comprises at least one immunoglobulin having a variable region comprising a CDR1, a CDR2, or a CDR3 region (page 22, lines 23-24), the at least one immunoglobulin having at least one protein fragment or peptide inserted within the variable region (page 22, lines 19 – 20); wherein (a) the protein fragment or peptide is GAD2 represented by SEQ. ID NO 4 (page 23, line 1), (b) the subject has undergone insulin autoantibody seroconversion prior to said administering step (page 21, line 10 and page 27 line 21- page 28, line 2) and (c) the composition is administered to the subject in one or more dosage administrations (page 37, lines 21-22 and original claim 1).

Although citations are given in accordance with 37 C.F.R. § 41.37(v), these reference numerals and citations are merely examples of support in the specification for the terms used in this section of the Brief. There is no intention to suggest in any way that the terms of the claims are limited to the examples in the specification. As demonstrated by the references numerals and citations, the claims are fully supported by the specification as required by law. However, it is improper under the law to read limitations from the specification into the claims. Pointing out specification support for the claim terminology in accordance with Rule 41.37(v) does not in any way limit the scope of the claims to those examples from which they find support. Nor does this exercise provide a mechanism for circumventing the law precluding reading limitations into the claims from the specification. In short, the references numerals and specification citations are not to be construed as claim limitations or in any way used to limit the scope of the claims.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Whether claims 1-5, 7, 13, 15-19, 22-24 and 26-30 are unpatentable under 35 U.S.C. §112, first paragraph as containing subject matter which was not described in the specification in such way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

2. Whether claims 1-5, 7, 13, 15-19, 22-24, and 26-30 are unpatentable under the judicially created doctrine of obviousness type double patenting over claims 1-7 and 13-16 of U.S. Patent Application No. 11/290,070.

3. Whether claims 1-5, 7, 13, 15-19, 22-24 and 26-30 are unpatentable under the judicially created doctrine of obviousness type double patenting over claims 1-7 and 13-16 of U.S. Patent Application No. 11/425,084.

4. Whether claims 1-5, 7, 13, 15-19, 22-24 and 26-30 are unpatentable under 35 U.S.C. §112, first paragraph as failing to comply with the written description requirement.

5. Whether claims 1, 2, 4, 5, 7, 13, 15-19, 22-24, 26 and 28-30 are unpatentable under 35 U.S.C. § 103(a) as being obvious over WO 98/30706 in view of Chao *et al.* (1999) *PNAS* 96:9299-9304 ("*Chao*"). Copies of WO 98/30706 and *Chao* are attached hereto as EXHIBITS B and C, respectively, in the Evidence Appendix.

VII. ARGUMENT

1. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A PRIMA FACIE CASE OF LACK OF ENABLEMENT OR APPELLANT HAS REBUTTED ANY SUCH PRIMA FACIE CASE.

Claims 1-5, 7, 13, 15-19, 22-24 and 26-30 stand rejected under 35 U.S.C. §112, first paragraph as allegedly containing subject matter which was not described in the specification in such way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. As described in detail below, no *prima facie* case of lack of enablement has been established. Furthermore, even if a *prima facie* case of lack of enablement has been established, which is denied, Appellants previously rebutted it.

It is settled law that a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enablement requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein. MPEP 2164.04 citing *In re Marzocchi*, 58 C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. (BNA) 367, 369 (CCPA 1971). Thus, the PTO has the initial burden of challenging a presumptively correct assertion of enablement in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *See In re Bundy*, 642 F.2d 430, 433, 209 U.S.P.Q. (BNA) 48, 51 (CCPA 1981).

The Examiner has alleged for various reasons that the instant claims are not enabled by the present disclosure. In particular, the Examiner purports that the state of the art would require undue experimentation for administering peptides to induce immune tolerance to prevent/delay the onset of Type 1 diabetes in humans because such methods were unpredictable at the time of the present invention. However, the PTO has not provided any credible evidence showing that one of ordinary skill in the art would ***reasonably doubt*** the asserted utility of the claimed

invention and has therefore not met its initial burden. The final Office Action dated July 29, 2011 (EXHIBIT A) at page 2 states that:

> [w]hile the mechanism of action for the method of the instant claims is not disclosed, it appears to require inducing tolerance to GAD and altered GAD 'derived' peptides. Tolerance-inducing peptide immunotherapy is well known in the immunological arts. In some cases significant results have been demonstrated in in-bred small animal models. However said results have not been repeated in human trials.

As an initial matter, Appellants point out that tolerance induction as a mechanism of action is not a limitation of the present claims. Furthermore, as discussed in detail below, the sum total the evidence provided by the Patent Office shows that, using fundamentally different therapeutic agents than presently claimed, tested in diseases other than Type 1 diabetes as presently claimed, some researchers have achieved tolerance results in animal models that have been difficult to reproduce in humans. See, Final Office Action dated July 29, 2011 at page 3 (EXHIBIT A). At most, these references are only tangentially related to the presently claimed invention and simply do not cast any doubt, let alone any reasonable doubt, on the presently clamed invention which entails an altogether different therapeutic agent and altogether different disease state than those discussed in the references relied upon by the PTO. This is simply not enough to establish a *prima facie* case of lack of enablement.

Based on the logic used in the instant rejection, the use of a novel compound to treat a given cancer would be unpatentable in the absence of human data if prior treatment of an altogether different cancer with a different compound had shown success in animals but failed to achieve FDA approval. This is neither the law nor sound policy. Appellants respectfully submit that the burden of challenging the presumptively correct assertion of the manner of making and using the invention has not been met.

A. No *Prima Facie* Case of Lack of Enablement has been Established.

i. Post-filing Date References Cannot Be Used in an Enablement Rejection.

MPEP 2164.05(a) and *In re Hogan* (559 F.2d 595, 605, 194 USPQ 527, 537 (CCPA 1977)) make clear that post-filing date references should not be used to demonstrate that the patent is non-enabling. Two narrow exceptions to this rule exist in cases in cases where (1) a

later-dated reference provides evidence of what one skilled in the art would have known **on or before** the effective filing date of the patent application or (2) if a later-dated reference actually **discloses the claimed invention**. *Id.*

In the Final Office Action mailed July 29, 2011 (EXHIBIT A), the Examiner attempts to overturn years of existing case law by setting forth that any post-filing date reference can be used in an enablement rejection. Specifically, the Examiner suggests that a post-filing date reference that provides evidence of what one skilled in the art learned after the filing date can be used in an enablement rejection. This is not the law. The MPEP says that, 'If individuals of skill in the art state that a particular invention was not possible years after the filing date, that would be evidence that the disclosed invention was not possible at the time of filing and should be considered." The phrase "particular invention" clearly refers to the claimed invention for which enablement is being challenged—referred to in the MPEP as the "disclosed invention." The two examples cited by the Examiner reinforce this conclusion. In other words, in order to fall within the exception, the post-filing date references must disclose an invention that falls within the scope of the claimed invention for which enablement is being challenged—*i.e.* the same invention.

In *In re Wright,* (MPEP 2164.06(b)(B)) the patentee described a process for the production of a recombinant vaccine which conferred immunity against the RNA tumor virus Prague Avian Sarcoma Virus (PrASV) but sought to patent claims directed to processes for producing recombinant vaccines against "all RNA viruses as well as avian RNA viruses." (emphasis added). The Court in *Wright* held that such claims were not enabled in view of a reference dated 5 years after the filing date of Wright's application – the reference disclosing that recombinant vaccines to AIDS retroviruses (an RNA virus) did not produce antibodies that could prevent viral infectivity. Because the claims broadly covered processes for producing recombinant vaccines against "all RNA viruses" and the post-filing date reference disclosed that recombinant vaccines against one particular RNA virus (AIDS retrovirus) did not produce antibodies that could prevent viral infectivity, enablement was lacking. In other words, the post-filing date reference disclosed an unsuccessful embodiment that was within the scope of the claimed genus of RNA viruses (*i.e.* the claimed invention).

In the second case cited by the Examiner, *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993), the 1985 application enabled protein expression in dicotyledonous plant

cells, but the claims were broadly directed to protein expression in any plant cell. See MPEP 2164.06(b)(C). The PTO provided evidence that as of 1987, the use of the claimed method in monocot plant cells was not enabled. Again, the post-filing date reference disclosed an unsuccessful embodiment falling within the scope of the claims at issue (*i.e.* the claimed invention).

In the present case, none of the references relied on by the Examiner disclose an embodiment falling within the scope of the present claims and most or all are only tangentially related to the presently claimed invention. As such, these post-filing date references clearly do not fall into the narrow exception to the rule, set forth in *Hogan*, that post-filing date references should not be used to demonstrate that the patent is non-enabling unless they actually disclose the claimed invention. Accordingly, the post-filing date references cited by the Examiner cannot be used to reject to the instant claims for lack of enablement because the claimed invention is not disclosed in any of the references cited by the PTO.

The references being relied on for lack of enablement are as follows (publication year in parentheses):

1) Legge (1998) (EXHIBIT D), entered in the record in Non-Final Office Action mailed August 24, 2007;

2) Marketletter (1999) (EXHIBIT E), entered in the record in Non-Final Office Action mailed August 24, 2007;

3) Dong (1999) (EXHIBIT F), entered in the record in Non-Final Office Action mailed August 24, 2007;

4) Pozzilli (2000) (EXHIBIT G), entered in the record in Non-Final Office Action mailed April 15, 2010;

5) Goodnow (2001) (EXHIBIT H), entered in the record in Non-Final Office Action mailed April 15, 2010;

6) WO 02/053092 (July 2002) (EXHIBIT I), entered in the record in Non-Final Office Action mailed April 15, 2010;

7) Skyler (2005) (EXHIBIT J), entered in the record in Non-Final Office Action mailed April 15, 2010;

8) Kraus and Mayer (2005) (EXHIBIT K), entered in the record in Non-Final Office Action mailed April 15, 2010;

9) Leslie (2010) (EXHIBIT L), entered in the record in Non-Final Office Action mailed April 15, 2010;

10) Bell (2008) (EXHIBIT M), entered in the record in Non-Final Office Action mailed April 15, 2010;;

11) von Herrath and Nepom (2009) (EXHIBIT N), entered in the record in Non-Final Office Action mailed April 15, 2010; and

12) Van der Worp (2010) (EXHIBIT O), entered in the record in Non-Final Office Action mailed April 15, 2010.

Each of references 6-12 were published **after** the priority date of the present application and are impermissibly being relied on for what one skilled in the art would have known **on or before** the effective filing date of the patent application. Moreover, none of references 6-12 discloses the claimed invention and thus do not fall within the narrow exception set forth in Hogan. As such, these references should not be used in an enablement rejection.

For at least the foregoing reasons, no *prima facie* case of lack of enablement has been established. Reversal of this rejection is therefore respectfully requested.

ii. Pre-filing date references do not establish a *prima facie* case of lack of enablement.

As has been extensively discussed in the record, none of the pre-filing date references establish a *prima facie* case of lack of enablement. It is settled law that a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enablement requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein. MPEP 2164.04 citing *In re Marzocchi*, 58 C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. (BNA) 367, 369 (CCPA 1971). Thus, the PTO has the initial burden of challenging a presumptively correct assertion of enablement in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility

does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *See In re Bundy*, 642 F.2d 430, 433, 209 U.S.P.Q. (BNA) 48, 51 (CCPA 1981).

The PTO has not provided any credible evidence showing that one of ordinary skill in the art would ***reasonably doubt*** the asserted utility of the claimed invention and has therefore not met its initial burden. Each of the pre-filing date references are addressed below:

- *Marketletter*; and *Pozzilli*

The Examiner alleges that practice of the instant claims would require undue experimentation because attempts to induce immune tolerance in humans have been unsuccessful as supported by *Marketletter* (EXHIBIT E) and *Pozzilli* (EXHIBIT G) which allegedly disclose failed experiments involving the use of peptides to induce immune tolerance. However, Appellants reiterate that *Marketletter* and *Pozzilli* simply do not disclose the administration of a fusion protein construct, let alone a fusion protein construct comprising GAD2 to as presently claimed. Specifically, *Marketletter* is a review of free peptides in multiple sclerosis and rheumatoid arthritis and *Pozzilli* evaluated insulin administration in diabetes. These references provide no evidence that a person of ordinary skill in the art at the time of filing of the instant application would have reasonably doubted the asserted utility of the presently claimed invention.

- *Dong*; *Legge*; and *Goodnow*

Additionally, the Examiner alleges that practice of the instant claims would require undue experimentation because *Dong,* (EXHIBIT F), *Legge* (EXHIBIT D) and *Goodnow* (EXHIBIT H) disclose that immune tolerance is not predictable. Again, none of *Dong*, *Legge* or *Goodnow* contain anything to call in to question the use of a fusion protein comprising GAD2 (in the presently claimed construct) for preventing or delaying type 1 diabetes in a subject as claimed. *Dong* is merely a general review of tissue graft transplant tolerance (unrelated to treatment of any autoimmune disorder let alone type 1 diabetes) and also contains nothing to call in to question use of a fusion protein construct as claimed for preventing or delaying type 1 diabetes in a subject that has undergone insulin autoantibody seroconversion. Additionally, *Legge* discloses the use of Ig constructs comprising PLP-LR (a protein implicated in MS). Moreover, *Goodnow* is a general review article about pathways for self-tolerance in auto-immune disorders. Again, none of these reference contain anything to call in to question the use of a fusion protein

comprising the GAD2 peptide (in the presently claimed construct) for preventing or delaying the onset of type 1 diabetes.

At most, the references properly relied on by the Examiner are only tangentially related to the presently claimed invention and/or simply do not cast any doubt, let alone any reasonable doubt, on the presently clamed invention. This is simply not enough to establish a *prima facie* case of lack of enablement. Withdrawal of the rejection is respectfully requested.

B. The Application Clearly Teaches How to Make and Use the Claimed Invention as is Required for Enablement.

Again, the enablement requirement consists of two prongs: first, the application must describe how to make the invention; second, the application must describe how to use the invention. 35 U.S.C., paragraph 1. The instant application clearly meets both prongs.

The instant claimed method is directed to preventing or delaying onset of Type 1 diabetes in a subject by, *inter alia*, administering to the subject a pharmaceutically acceptable composition comprising a soluble fusion protein comprising at least one immunoglobulin having at least one variable region and at least one peptide inserted within the at least one variable region, wherein the at least one peptide is GAD2 represented by SEQ ID NO: 4. The application contains everything need for the person of ordinary skill in the art to make and use the claimed invention.

Specifically, guidance as to how to make the claimed constructs is provided in the specification at pages 45, line 13 - page 47, line 3. General dosing guidance is provided in the specification at page 34, line 21- page 37, line 17. Guidance for determining whether administration of a claimed fusion protein effectively prevented or delayed diabetes in humans or mice is provided in the specification at page 42, line 3 - page 42, line 7 and page 45, lines 5-11. Guidance for determining if a subject has undergone insulin autoantibody seroconversion is provided in the specification at page 55, line 15 – page 56, line 24.

C. Even assuming, *arguendo,* that a *prima facie* case exists, Appellants previously rebutted it.

To further demonstrate that Appellants' claimed invention was enabled at the time of filing, Appellants previously submitted a declaration under 37 CFR 1.312 showing that the claimed method effectively prevents and/or delays the onset of Type 1 diabetes in the gold standard NOD mouse model for that disease. ("Zaghouani Declaration I") (EXHIBIT P). As has

been clearly established in the record, the NOD mouse model used in the experiment described in the Zaghouani Declaration is considered the gold standard animal model for Type 1 diabetes, regardless of whether some unrelated agent in the past has shown efficacy in that model that did not translate to humans. The pharmaceutical industry is replete with molecules that showed efficacy in gold standard models that failed to achieve FDA approval—this does not render later, different candidate molecules unpatentable for lack of enablement.

It was clear error to reject the instant claims under 35 U.S.C. § 112, first paragraph on the alleged basis that successful results in the gold standard animal model for Type 1 diabetes do not necessarily translate to humans or other species. Appellants have taught the public that the claimed soluble IgGAD2 construct can prevent or delay onset of Type 1 diabetes in a standard experimental animal and have thus made a significant and useful contribution to the art, even though it could eventually be determined that the compound is without value in the treatment of humans. These data demonstrate that the claimed invention was enabled when filed. MPEP 2164.05 and *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995).

i. Treatment of humans is not required.

The Examiner further takes the position that while tolerance-inducing peptide immunotherapy is well known in the immunological arts and has shown significant results in in-bred small animal models – the results have not been repeated in human trials. EXHIBIT A at page 2. The Examiner attempts to support this conclusion by citing to instances in which treatment (albeit again with fundamentally different types of therapeutic agents and in different diseases than Type 1 diabetes as discussed above) succeeded in animals but failed in humans—for example *Marketletter*. That position—also rejected by the Federal Circuit in *Brana*—is clear legal error.

In *Brana*, the PTO argued in the context of a 112, first paragraph rejection that *in vivo* test results in animals are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. *Id* at 20. In response, the Federal Circuit stated:

> The Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption... proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. *In re Krimmel*, 48 C.C.P.A. 1116, 292 F.2d 948, 953...In concluding that similar *in vivo* tests were adequate proof of utility the court in *In re Krimmel* stated: We hold as we do

> because it is our firm conviction that one who has taught the public that a compound exhibits some desirable pharmaceutical property **in a standard experimental animal has made a significant and useful contribution to the art, even though it may eventually appear that the compound is without value in the treatment of humans**. *Id.* At 22. (emphasis added)

Again, the NOD mouse model used in the experiment described in the Zaghouani Declaration I (See, EXHIBIT P) is considered the gold standard animal model for Type 1 diabetes. It was clear error to reject the instant claims under 35 U.S.C. § 112, first paragraph on the alleged basis that successful results in the gold standard animal model for Type 1 diabetes do not necessarily translate to humans or other species. Appellants have taught the public that the claimed soluble IgGAD2 construct can prevent or delay onset of Type 1 diabetes in a standard experimental animal and have thus made a significant and useful contribution to the art, even though it could eventually be determined that the compound is without value in the treatment of humans.

For at least the foregoing reasons, no *prima facie* case of lack of enablement has been established. Even if a *prima facie* case of lack of enablement is deemed to have been established, which is not admitted, Appellants have rebutted it. Reversal of this rejection is therefore respectfully requested.

2. REJECTION UNDER THE JUDICIALLY CREATED DOCTRINE OF OBVIOUSNESS TYPE DOUBLE PATENTING OVER CLAIMS 1-7 AND 13-16 OF U.S. 11/290,070 SHOULD BE WITHDRAWN.

Appellants respectfully note that 37 CFR § 41.37 does not require that "all pending rejections" be addressed. Rather, 37 CFR § 41.37 specifies that the appeal brief shall provide a concise statement of "each ground of rejection *presented for review*." Appellants submit that the instant double patenting rejection is a *provisional* rejection only. As is made clear by MPEP 804.2, if a provisional obviousness-type double patenting rejection between two pending applications is the only rejection remaining in the earlier filed of the two applications, the Examiner should withdraw the rejection in the earlier filed application and permit that application to issue as a patent without a terminal disclaimer. The instant application was filed prior to U.S. 11/290,070. Therefore, assuming Appellants have overcome all other outstanding

rejections in this application, the instant provisional rejection should be withdrawn as a matter of course and the instant application allowed to issue.

3. REJECTION UNDER THE JUDICIALLY CREATED DOCTRINE OF OBVIOUSNESS TYPE DOUBLE PATENTING OVER CLAIMS 1-7 AND 13-16 OF U.S. U.S. 11/425,084 SHOULD BE WITHDRAWN.

Appellants note that 37 CFR § 41.37 does not require that "all pending rejections" be addressed. Rather, 37 CFR § 41.37 specifies that the appeal brief shall provide a concise statement of "each ground of rejection *presented for review*." Appellants submit that the instant double patenting rejection is a *provisional* rejection only. As is made clear by MPEP 804.2, if a provisional obviousness-type double patenting rejection between two pending applications is the only rejection remaining in the earlier filed of the two applications, the Examiner should withdraw the rejection in the earlier filed application and permit that application to issue as a patent without a terminal disclaimer. The instant application was filed prior to U.S. 11/425,084. Therefore, assuming Appellants have overcome all other outstanding rejections in this application, the instant provisional rejection should be withdrawn as a matter of course and the instant application allowed to issue.

4. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH AS FAILING TO COMPLY WITH THE WRITTEN DESCRIPTION REQUIREMENT SHOULD BE REVERSED BECAUSE THE EXAMINER FAILED TO ESTABLISH A PRIMA FACIE CASE.

Claims 1-5, 7, 13, 15-19, 22-24 and 26-30 stand rejected under 25 U.S.C. § 112, first paragraph as failing to comply with the written description requirement. In order to establish a *prima facie* case of lack of written description, the Examiner must show that the application as filed does not reasonably describe or convey to one of ordinary skill in the art, at the time of filing the application, that the inventor had possession of the claimed invention. MPEP § 2163.03. As stated by the Board, "the examiner has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in [the] specification disclosure a description of the invention defined by the claims. *Ex parte Sorenson*, 3 U.S.P.Q.2d 1462 (BPAI 1987). "It is not necessary that the application describe the claim limitations exactly,...but only

so clearly that persons of ordinary skill in the art will recognize from the disclosure that appellants invented processes including those limitations." *In re Wertheim*, 541 F.2d 257 (CCPA 1976). Adequate description under the first paragraph of 35 U.S.C. § 112 does not require literal support for the claimed invention...Rather, it is sufficient if the originally-filed disclosure would have ***conveyed to one having ordinary skill in the art*** that an applicant had possession of the ***concept*** of what was claimed. *Ex parte Parks*, 30 U.S.P.Q.2d 1234 (BPAI 1994).

In the Final Office Action mailed July 29, 2011 (EXHIBIT A), the Examiner states that the specification and originally filed claims do not provide support for the invention as now claimed, specifically: (A) a method comprising administration of an immunoglobulin construct comprising a protein represented by SEQ ID NO: 4 (claims 1 and 13). As will be discussed in detail below, no *prima facie* case of lack of written description has been established and this rejection should be reversed.

Claims 1 and 13 each claim a method of preventing or delaying onset of Type 1 diabetes comprising, *inter alia*, administering to a subject a soluble fusion protein comprising at least one immunoglobulin having at least one protein fragment or peptide inserted in the variable region, wherein the protein fragment or peptide is (claim 1), or consists essentially of (claim 13), GAD2 represented by SEQ ID NO: 4.

Appellants point out that the specification as filed contains multiple descriptions of an immunoglobulin construct comprising GAD2/SEQ ID NO: 4 for preventing or delaying onset of Type 1 diabetes. For example and without limitation the instant specification provides:

- "The present invention is directed to methods, compounds, compositions, combinations, and kits for treating, preventing, suppressing or delaying the onset, or reducing the risk of developing type 1 diabetes, or the symptoms associated with, or related to, type 1 diabetes, in a subject in need thereof. In one aspect, the present invention is directed to compounds, compositions, kits, and methods for endocytic presentation of an immunosuppressive factor for the down regulation of diabetogenic T cells for the treatment or prevention of type 1 diabetes. In yet another embodiment of the present invention, methods, kits, combinations, and compositions containing at least one immunoglobulin, for example, INS, GAD, an insulin protein, a peptide derived from insulin, a diabetogenic epitope, or a T cell receptor engaging determinant, are provided to treat, prevent, suppress, or delay the onset of type 1 diabetes after expression of an IAA predisposition marker." Page 4, lines 11-21.

- "1) A method for the treatment of type 1 diabetes in a patient during the pre-insulitis stage of diabetes by administration of a composition comprising an

immunoglobulin or portion thereof linked to a peptide wherein the immunoglobulin or portion thereof is aggregated." Page 6, lines 13-15. "21) The method of paragraph 1 wherein the peptide is selected from the group consisting of GAD1 and GAD2." Page 8, lines 7-8.

- "The present invention is also directed to a methods, kits, combinations, and compositions, comprising: a pharmaceutically-effective amount of an immunoglobulin, or portion thereof, linked to a protein fragment or peptide, wherein the immunoglobulin, or portion thereof, can bind to an Fc receptor. Illustratively, the peptide comprises INSβ, GAD 1, or GAD2." Page 19, lines 1-5.

- "In yet another embodiment of the present invention, the immunoglobulin comprises Ig-INSβ, Ig-GAD1, Ig-GAD2, or an immunoglobulin, or a portion thereof, linked to a peptide, for example a peptide derived from GAD65 or an insulin protein." Page 22, lines 3-5.

- "In yet another embodiment of the present invention, the composition comprises IgINS (peptides derived from human insulin), IgGAD (peptides derived from GAD), IgINSβ, IgGAD1 and IgGAD2." Page 23, lines 2-4.

- "In one embodiment of the present invention, the composition comprises Ig-INSβ, Ig-GAD1, IgGAD2 or an immunoglobulin or a portion thereof linked to a peptide derived from GAD65." Page 24, lines 6-8.

- "In one embodiment of the present invention, a composition is provided comprising an immunoglobulin or portion thereof linked to a protein fragment or peptide wherein the immunoglobulin or portion thereof is capable of binding to an Fc receptor, the peptide being selected from the group consisting of peptides derived from INS and GAD and more specifically INSβ, GAD 1 and GAD2, the composition having the property of being endocytosed by cells bearing the Fc receptor and processed and presented by the cells to present the peptide to endogenous MHC Class II molecules, thereby substantially reducing or preventing activation of diabetogenic T cells specific for the peptide." Page 24, lines 14-21.

- "Other peptides that may be inserted within the variable region within the CDR region of an Ig and utilized for creating compositions for the treatment of type 1 diabetes as taught in the present invention are: GAD1 (Glutamic acid decarboxylase-65 also known as 'GAD65'); corresponding to amino acid residues 524-543 of GAD 65 (Seq. I.D. No. 3 [SRLSKVAPVIKARMMEYGT]) to create chimera Ig-GAD1; and 2) GAD2; corresponding to amino acid residues 206-220 of GAD 65 (Seq. I.D. No. 4 [TYEIAPVFVLLEYVT]); and other peptides derived from GAD65." Page 45, line 20, to page 46, line 2 (bracketed text appears in original).

In view of the above excerpts from the instant specification, Appellants note that the specification as filed clearly provides written support for a method of preventing or delaying onset of Type 1 diabetes comprising administration of an immunoglobulin construct comprising a protein represented by SEQ ID NO: 4.

Because the originally filed disclosure would have conveyed to one having ordinary skill in the art that the Appellants had possession of the concept of what is being claimed, the instant written description rejection should be reversed.

5. REJECTION UNDER 35 U.S.C. §103(A) SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A *PRIMA FACIE* CASE OF OBVIOUSNESS.

Claims 1, 2, 4, 5, 7, 13, 15-19, 22-24, 26 and 28-30 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over WO 98/30706 (EXHIBIT B) in view of Chao *et al.* (1999) *PNAS* 96:9299-9304 ("*Chao*") (EXHIBIT C). As will be discussed in detail below, no *prima facie* case of obviousness has been established.

To establish a *prima facie* case of obviousness under 35 U.S.C. § 103, the Office must articulate a reason or rationale that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does. See, *e.g.*, *KSR* 550 U.S. 398 (2007); *Omegaflex, Inc. v. Parker-Hannifin Corp.*, 243 Fed. App'x. 592, 595-596 (Fed. Cir. 2007) citing *KSR*. Further, the Supreme Court in *KSR* also stated that that "a court *must* ask whether the improvement is more than the predictable use of prior art elements according to their established functions." *KSR* at 1740; emphasis added.

Where the rationale used by the PTO to reject claims as obvious is based on some alleged teaching, suggestion, or motivation in the prior art that would have led one of ordinary skilled in the art to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention, the PTO must articulate the following:

(1) a finding that there was some teaching suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings;

(2) a finding that there was a reasonable expectation of success; and

(3) whatever additional findings based on the Graham factual inquiries may be necessary in view of the facts to explain a conclusion of obviousness. See MPEP 2143(G).

According to the Final Office Action mailed July 29, 2011 (See, EXHIBIT A), WO 98/30706 (EXHIBIT B) teaches the treatment of autoimmune disorders employing a humanized IgG2b chimeric protein wherein an autoantigen peptide is inserted into the D segment of a CDR3 loop. EXHIBIT C at page 9. WO 98/30706 is silent as to GAD65, GAD1 and GAD2. *Chao*, on the other hand, is cited for its disclosure that the GAD65 peptide of SEQ ID NO: 4 is an immunodominant T-cell diabetes antigen in a NOD mouse model. EXHIBIT C at page 9300. The Examiner therefore concludes that it would have been obvious to insert the full length GAD65 protein of *Chao* into a construct of WO 98/30706 and that such a person would have had a reasonable expectation of preventing or delaying the onset of Type 1 diabetes at the priority date of the instant application. As such, the instant obviousness rejection is based on a "teaching, suggestion, or motivation" rationale—so the PTO's argument goes, since GAD65 was a known Type 1 diabetes autoantigen at the time of filing, one of skill in the art would have been motivated to insert it into the construct of WO 98/30706 and would have had a reasonable expectation of preventing or delaying the onset of Type 1 diabetes.

A. No reasonable expectation of success.

Even if one of ordinary skill in the art would have had some reason combine the construct of WO 98/30706 (EXHIBIT B) with the GAD65 peptide (SEQ ID NO: 4) of *Chao* (EXHIBIT C) to prevent or delay the onset of type 1 diabetes, which is not admitted, such a person would not have had a reasonable expectation of success in preventing or delaying the onset of type 1 diabetes, particularly in a subject that had undergone insulin autoantibody seroconversion.

The Examiner relies on WO 98/30706 (EXHIBIT B) which discloses a fusion protein having the proteolipid protein (PLP) autoantigen inserted into the D segment of a CDR3 loop. EXHIBIT A at page 10. PLP is an autoantigen associated with multiple sclerosis. Appellants respectfully submit that the multiple sclerosis test model used in WO 98/30706 (experimental allergic encephalomyelitis) is far different from the type 1 diabetes NOD mouse model used in examples within the instant application such that any success or failure shown in WO 98/30706 would not be at all predictive of success or failure of an Ig-GAD2 fusion protein in prevention or delay of type 1 diabetes as presently claimed.

Specifically, the relevant examples in WO 98/30706 (*e.g.* Examples I and XI) involve induction of an immune response with a known pathogenic peptide (PLP1) followed by treatment of the induced immune response with a slightly altered version of the very same peptide (PLP-LR) introduced in the form of a chimeric antibody immunomodulating agent. PLP-LR is an analog of PLP1 in which Trp144 and His147 are replaced with Leu and Arg, respectively. Therefore, in Examples I and XI of WO 98/30706, a disease state is induced with a known pathogenic peptide and then treated with a slightly altered non-pathogenic version of the very same peptide.

In stark contrast to those examples, the onset of type 1 diabetes in the NOD mouse model is a *spontaneous* event not triggered by administration of a known peptide antigen. Because no inducer peptide is known or administered, it was completely unpredictable at the time the present invention was made which peptide antigen, if any, when incorporated into compositions disclosed in the instant application, would have any impact on type 1 diabetes, let alone delay or prevent that disease state. This is very different from the situation in WO 98/30706 in which the disease inducing peptide was known at the outset, and treatment was provided with a slight variation of the very same inducer peptide. In view of these significant differences and the highly unpredictable area of art of the presently claimed invention, a person of ordinary skill in the art at the time the present invention was made would not have had a reasonable expectation of success in delaying or preventing type 1 diabetes according to the presently claimed methods. Appellants respectfully submit that the outcome of the presently claimed methods was highly unpredictable at the time the present invention was made.

Furthermore, one of ordinary skill in the art at the time the present invention was made would not have had a reasonable expectation that the GAD65 peptide of *Chao* (SEQ ID NO: 4), selected from the numerous type 1 diabetes autoantigen peptides and protein fragments known or suspected at the time, would prevent or inhibit diabetes as presently claimed. There is no articulated rationale in the record for selection of any particular diabetogenic peptide or protein fragment, nor any indication why a person of ordinary skill in the art would have had a reasonable expectation of delaying or preventing type 1 diabetes in an IAA positive subject with any such peptide or protein fragment.

For at least the foregoing reasons, a person of ordinary skill in the art would not have had a reasonable expectation of success of preventing or delaying the onset of diabetes according to

the presently claimed methods. Withdrawal of the instant rejection is therefore respectfully requested.

B. Unexpected results.

It is well settled that "[o]ne way for a patent applicant to rebut a *prima facie* case of obviousness is to make a showing of 'unexpected results,' *i.e.*, to show that the claimed invention exhibits some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or unexpected." *In re Soni*, 54 F.3d 746, 750 (Fed. Cir. 1995). Additionally, as was articulated in *In re Soni*, "when an applicant demonstrates *substantially* improved results...and states that the results were unexpected, this should suffice to establish unexpected results in the absence of evidence to the contrary. 54 F.3d 746 at 751(Fed. Cir. 1995).

As is discussed in detail below, the presently claimed soluble Ig-GAD2 composition provides unexpected results in that it has the ability to rescue residual and form new insulin-producing β cells. Appellants point to an article co-authored by inventors Zaghouani and Gregg (Jain *et al.*, Innocuous IFNγ induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production, JEM Vol. 205, No. 1 (2008); hereinafter "Jain") (EXHIBIT Q) and declaration under 37 CFR 1.312 ("Zaghouani Declaration II") (EXHIBIT R), both previously made of record in Appellants' response dated May 20, 2011, setting forth unexpected properties of the soluble Ig-GAD2 construct.

Appellants respectfully direct the Board's attention to page 209 of EXHIBIT Q (section titled "Treatment with Ig-GAD2 increases the number of healthy pancreatic islets"). It will be noted that animals treated with the soluble Ig-GAD2 construct had significantly greater number of total islets than the hyperglycemic or diabetic mice ($P = 0.0001$). EXHIBIT Q, Figures 3B and 3 C. The number of insulin-positive islets also increased from 14 per pancreas at the prediabetic (hyperglycemic) stage to 29 per pancreas upon treatment with Ig-GAD2. Also, the 15-week treatment group had a higher number of islets with periinsulits (38% vs. 30%) or no insulitis (35% vs. 17%) relative to the hyperglycemic stage. EXHIBIT Q, Fig 3C. Overall, the treatment with Ig-GAD2 led to a significant increase in the number of non-inflamed (healthy) islets.

Moreover, as set forth starting at column 2, page 210 of *Jain* (EXHIBIT Q), an experiment was conducted to determine whether the increased number of healthy islets in the treated animals was caused by regression of cell infiltration and/or formation of new β cells. To

address this question, treated mice were injected with 100 mg/kg of the proliferation indicator BrdU and pancreas sections were double stained with anti-insulin and BrdU antibodies and analyzed for BrdU incorporation and insulin production. Because the relevant figures in *Jain* for this experiment include colored arrows, Appellants attach herewith for the Board's convenience annotated versions of these figures with labels indicating colors of the arrows (EXHIBIT S). Blue arrows indicated $BrdU^+$ cells, green arrows indicate $insulin^+$ cells, and red arrows indicated $BrdU^+/Insulin^+$ cells.

Appellants point out that BrdU staining was visible in the highly proliferative luminal intestinal cells used as a control, but these had no staining with anti-insulin antibody. EXHIBIT S, Fig. 4A, blue arrow. Islets of nondiabetic 5-wk old mice were positive for insulin, but did not incorporate BrdU, suggesting that these insulin-producing β cells were not dividing. EXHIBIT S, Fig. 4B, green arrow. Thus, under normal circumstances, insulin production emanates from existing β cells whose nuclei do not incorporate BrdU, giving a minimal number of BrdU/insulin double positive β cells ($BrdU^+/insulin^+$). The hyperglycemic mice showed very few insulin-producing β cells and no BrdU incorporation (EXHIBIT S, Fig. 4 C, green arrow), resulting in an insignificant number of $BrdU^+/insulin^+$) β cells (EXHIBIT S, Fig. 4 E). In contrast, as shown in Fig. 4 D (See, EXHIBIT S), islets from the 25-wk treatment group showed $insulin^+$ β cells that were either BrdU- (green arrows; residual β cells) or $BrdU^+$ (red arrows; newly formed β cells). The number of insulin-producing dividing β cells was significantly ($P = 0.0001$ for treated group compared to hyperglycemic group) increased in all 10 mice in which treatment restored normoglycemia. EXHIBIT S, Fig. 4 E. Error bars indicated the standard deviation of 10 pacreata.

Overall, Appellants submit that these results indicate that treatment with soluble Ig-GAD2 reduces cell infiltration, leading to rescue of residual and formation of new insulin producing β cells. Thus, even if the Examiner has demonstrated a *prima facie* case of obviousness, which Appellants do not admit, the unexpected results obtained with soluble Ig-GAD2 would overcome such a rejection of the instant claims. Accordingly, withdrawal of the instant rejection is respectfully requested.

C. Each and every claim limitation not disclosed in the prior art.

Claim 1 and all claims depending there from specify that "the subject has undergone insulin autoantibody seroconversion prior to the administering step." This limitation is simply

not disclosed in the prior art of record. Because the prior art when combined does not teach each and every limitation of the instantly claimed invention, the asserted prima facie case of obviousness fails.

For at least the foregoing reasons, no *prima facie* case of obviousness has been established and reversal of the instant obviousness rejection is respectfully requested.

VIII. CONCLUSION

Appellants respectfully submit that the Examiner has failed to: (1) establish a *prima facie* case of lack of enablement or alternatively any such *prima facie* case has been rebutted; (2) establish a *prima facie* case of lack of written description; and (3) establish a *prima facie* case of obviousness. Accordingly, Appellants respectfully submit that these rejections are erroneous in law and in fact and should therefore be reversed by this Board.

The Director is authorized to charge any fees that may be required, or to credit any overpayment to Deposit Account No. 02-1818. If such a withdrawal is made, please indicate the Attorney Docket No. 3718027.00005 on the account statement.

Respectfully submitted,

K&L GATES LLP

BY _____

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Dated: January 30, 2012

CLAIMS APPENDIX

PENDING CLAIMS ON APPEAL OF U.S. PATENT APPLICATION SERIAL NO. 10/681,788

1. A method of preventing or delaying onset of Type 1 diabetes in a subject in need thereof, the method comprising administering to the subject a pharmaceutically acceptable composition comprising a soluble fusion protein, wherein the fusion protein comprises at least one immunoglobulin having a variable region comprising a CDR1, a CDR2, or a CDR3 region, the at least one immunoglobulin having at least one protein fragment or peptide inserted within the variable region; wherein (a) the protein fragment or peptide is GAD2 represented by SEQ. ID NO 4, (b) the subject has undergone insulin autoantibody seroconversion prior to said administering step and (c) the composition is administered to the subject in one or more dosage administrations.

2. The method of claim 1, wherein the immunoglobulin is human or humanized.

3. The method of claim 1, wherein the subject is a human subject.

4. The method of claim 1, wherein administration of the composition to the subject results in down regulation of an autoreactive T cell.

5. The method of claim 1, wherein the at least one protein fragment or peptide is inserted within a CDR region of the at least one immunoglobulin.

7. The method of claim 5, wherein administration of the composition to the subject results in substantially reduced activation of an autoreactive T cell specific for the at least one protein fragment or peptide.

8. The method of claim 1, wherein the at least one protein fragment or peptide is derived from INS.

9. The method of claim 8, wherein the INS comprises soluble INSß.

10. The method of claim 9, wherein the soluble INSß is capable of binding to at least one Fc receptor.

11. The method of claim 10, wherein the Fc receptor is a Fcγ receptor.

12. The method of claim 10, wherein the composition is capable of being endocytosed by antigen presenting cells.

13. The method of claim 1, wherein the at least one protein fragment or peptide consists essentially of GAD2 represented by SEQ. ID NO 4.

15. The method of claim 13, wherein the subject is GAD positive.

16. The method of claim 1, wherein the subject has not developed hyperglycemia at initiation of the administering step.

17. The method of claim 1, wherein the subject expresses a Type 1 diabetes predisposition marker at initiation of the administering step.

18. The method of claim 1, wherein upon administration of the composition to the subject, the subject undergoes a dose dependent suspension, prevention, or delay in onset of Type 1 diabetes.

19. The method of claim 1, wherein administration of a first dosage of the composition occurs before the subject has developed type-1 diabetes.

20. A composition for suppressing the onset of Type 1 diabetes in a subject that has undergone IAA seroconversion, the composition comprises: a_pharmaceutically acceptable composition comprising at least one immunoglobulin selected from the group consisting of INS, GAD, an insulin protein, a peptide derived from insulin, a diabetogenic epitope, and a T cell receptor engaging determinant.

21. The method of claim 20 wherein the fusion protein is in soluble form.

22. The method of claim 2 wherein the immunoglobulin is selected from the group consisting of IgG1, IgG2, IgG2a, IgG2b, IgG3, IgG4, IgGA, IgA1, IgA2, IgGE, IgD, IgE, or IgM.

23. The method of claim 5 wherein the at least one protein fragment or peptide is inserted within the CDR3 region of the immunoglobulin.

24. The method of claim 23 wherein the at least one protein fragment or peptide is inserted within the CDR3 region of the immunoglobulin in place of a D segment.

25. The method of claim 13 wherein the at least one protein fragment or peptide consists of amino acid residues 524-543 of GAD65.

26. The method of claim 13 wherein the at least one protein fragment or peptide consists of amino acid residues 206-220 of GAD65.

27. The method of claim 13 wherein the subject is a human.

28. The method of claim 1 wherein the pharmaceutical composition further comprises at least one pharmaceutically acceptable carrier.

29. The method of claim 28 wherein the composition comprises an aqueous solution or suspension.

30. The method of claim 29 where the administering step is accomplished by injection or infusion.

EVIDENCE APPENDIX

EXHIBIT A: Final Office Action mailed July 29, 2011.

EXHIBIT B: WO 98/30706. Entered in the record in Non-Final Office Action mailed August 24, 2007.

EXHIBIT C: Chao *et al.* (1999) *PNAS* 96:9299-9304 ("*Chao*"). Entered in the record in Non-Final Office Action mailed November 22, 2010.

EXHIBIT D: Legge (1998). Entered in the record in Non-Final Office Action mailed August 24, 2007.

EXHIBIT E: Marketletter (1999). Entered in the record in Non-Final Office Action mailed August 24, 2007.

EXHIBIT F: Dong (1999). Entered in the record in Non-Final Office Action mailed August 24, 2007.

EXHIBIT G: Pozzilli (2000). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT H: Goodnow (2001). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT I: WO 02/053092 (July 2002). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT J: Skyler (2005). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT K: Kraus and Mayer (2005). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT L: Leslie (2010). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT M: Bell (2008). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT N: Von Herrath and Nepom (2009). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT O: Van der Worp (2010). Entered in the record in Non-Final Office Action mailed April 15, 2010.

EXHIBIT P: Zaghouani Declaration I. Entered in the record along with the Office Action response filed December 19, 2007.

EXHIBIT Q: Jain *et al.*, JEM Vol. 205, No. 1 (2008) ("*Jain*").Zaghouani Declaration. Entered in the record along with the Office Action response filed May 20, 2011.

EXHIBIT R: Zaghouani Declaration II. Entered in the record along with the Office Action response filed May 20, 2011.

EXHIBIT S: Annotated figures from *Jain*. Entered in the record along with the Office Action response filed May 20, 2011.

RELATED PROCEEDINGS APPENDIX

None

EXHIBIT A

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/681,788	10/08/2003	Habib Zaghouani	3718027.00005	6701

24573 7590 07/29/2011

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EXAMINER
EWOLDT, GERALD R

ART UNIT	PAPER NUMBER
1644	

NOTIFICATION DATE	DELIVERY MODE
07/29/2011	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

chicago.patents@klgates.com

Office Action Summary	Application No.	Applicant(s)
	10/681,788	ZAGHOUANI ET AL.
	Examiner	Art Unit
	G. R. Ewoldt, Ph.D.	1644

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *20 May 2011*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-5,7-13 and 15-30* is/are pending in the application.

4a) Of the above claim(s) *8-12,20,21 and 25* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-5,7,13,15-19,22-24, and 26-30* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)	
1) ☐ Notice of References Cited (PTO-892)	4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)	5) ☐ Notice of Informal Patent Application
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.	6) ☐ Other: _____.

DETAILED ACTION

1. Applicant's remarks and 1.132 declaration of Inventor Zaghouani filed 5/20/11 are acknowledged.

2. Claims 8-12, 20, 21, and 25 stand withdrawn from further consideration by the examiner, 37 CFR 1.142(b), as being drawn to non-elected inventions.

Claims 1-5, 7, 13, 15-19, 22-24, and 26-30 are under examination.

3. The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

4. Claims 1-5, 7, 13, 15-19, 22-24, and 26-30 stand rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Specifically, the specification provides insufficient evidence that the claimed method could effectively function as a method for preventing or delaying the onset of type I diabetes (IDDM) in humans.

As set forth previously, While the mechanism of action for the method of the instant claims is not disclosed, it appears to require inducing tolerance to a GAD peptide. Tolerance-inducing peptide immunotherapy is well known in the immunological arts. In some cases significant results have been demonstrated in in-bred small animal models. However, said results have not been repeated in human trials. See for example, *Marketletter* (9/13/99) which teaches the complete failure in human trials of two peptides designed for tolerance induction. Both Myloral (for multiple sclerosis, MS) and Colloral (for rheumatoid arthritis, RA) provided successful results in rodent models (EAE and collagen induced arthritis, respectively). See also Leslie (2010) paraphrasing an interview with Dr. Mark Davis wherein Dr. Davis states that in the case of the administration of MBP for tolerance induction to MBP for the treatment of MS, while the method worked in mice, it actually made MS worse in some humans.

More specifically regarding the treatment of diabetes, see Pozzilli et al. (2000) wherein the authors demonstrate that, while the induction of tolerance to orally administered insulin for the treatment of diabetes might have been expected, it simply did not occur. The authors could only speculate as to the

reasons for the trial's failure. The authors did note one complicating factor that has been reported several times, and will have to be considered in all future work, a large placebo effect wherein both the treated and control subjects showed similar temporary improvement. Three years later Skyler et al. (2005) reported another failure in one of the largest placebo-controlled tolerance trials ever performed in humans (the administration of insulin for the prevention of type 1 diabetes).

As set forth above, the references demonstrate that peptides that work to induce immune tolerance in *in vivo* small animal disease models cannot be routinely expected to work in humans, i.e., they are unpredictable and requiring of undue experimentation.

Other investigators have discussed additional problems in establishing human tolerance. See, for example, Dong et al. (1999):

> "Despite the fact that it has been relatively easy to induce true tolerance in small experimental animals, translating these studies into larger animals and humans has been much more difficult to achieve. Some of the hurdles that may explain this dilemma are summarized in Table 3. *Even if we have the ideal strategy to use in humans, the lack of reliable predictable assays for rejection or tolerance still does not allow us to know if a patient is truly tolerant so that immunosuppressive agents may be withdrawn*",

emphasis added.

WO 02/053092 teaches that the oral administration of antigens (a route of administration encompassed by the claimed method) for the induction of tolerance presents numerous additional "obstacles", including the problem of accurate dosing given the necessity of digestion which alters both concentration and structure of the antigens. In that work the inventors conclude that:

> "oral and mucosal tolerance cannot be deduced from antigenic activity in conventional immunization, or even *in vitro* results, and must result from extensive empirical experimentation,"

In another attempt to explain these repeated failures Goodnow (2001) states:

> "Obtaining the desired response [tolerance] with these strategies [tolerance induction] is unpredictable because many of these signals [tolerogenic] have both tolerogenic and immunogenic roles,"

(see the Abstract). The author goes on to teach that while the induction of oral tolerance might be considered "an attractive notion", the method has failed in humans because of the lack of understanding of the mechanisms involved (page 2120, column 2).

More recently, Kraus and Mayer (2005) looked at tolerance induction in inflammatory bowel disease (IBD). They reported the ease with which tolerance is induced in in-bred experimental mice and contrasted that with the difficulty in inducing tolerance in humans. Speculating on the reasons for the difference the authors considered a lack of dosing optimization but went on to report that *the mechanisms of tolerance induction in humans and mice appear to be fundamentally different.* Most importantly, Kraus and Mayer report a genetic component wherein many IBD patients and their family members appear to be *incapable* of becoming

tolerant to oral antigens because they lack the ability to generate the required T regulatory cells. If confirmed, this would mean that *no* tolerance induction regime could work in these patients.

Even more recent work has attempted to duplicate favorable results established in in-bred animal models in a more complex mouse model more realistic to the out-bred human population. See, for example, Bell et al. (2008). The authors employed F_1 hybrid mice (a cross between two in-bred strains) wherein they asked if toleragens that worked in the parent strains would induce tolerance in the crossed F_1 hybrid mice. Unfortunately the results showed that in one instance not only was tolerance not induced, but disease was actually exacerbated. Thus, the work serves as a clear demonstration that the induction of immune tolerance is far from predictable in anything other than carefully chosen in-bred experimental mouse strains.

Also note that Applicant has referred to the NOD mouse as the "gold standard" for diabetes research. Others, however, refer to the NOD mouse as the "workhorse" for diabetes research pointing out the model's limitations. See, for example von Herrath and Nepom (2009). And note that not even all NOD mouse strains are diabetes susceptible, e.g., NOD H-2^k and NOD DQ8 do not develop the disease. Also note that it is well-known that tolerance to GAD is **not** effective for the treatment of diabetes in another well-established diabetes model, the BB rat. Even more recently many scientists have begun to question the value of mouse data altogether. As pointed out by Mark Davis in a recent interview, mice make a "lousy model" for the human immune system. He refers to mice as a short-lived rodent who's immune system has adapted for scurrying around with its nose in the dirt (Leslie, 2010). Also very recently van der Worp et al. (2010) question the value of using animal data to predict the effectiveness of treatment strategies in human trials. As an example, the authors teach that of about 500 effective treatment strategies for stroke in experimental mice, just 2 have proven effective in humans. The authors cite numerous possible reasons for the failed translation of results, including insufficient statistical power, inadequate animal data, overoptimistic conclusions, flawed studies, and the use of animal models that do not reflect real disease in humans. Finally the reference teaches that neutral and negative animal studies may remain unpublished leading to possibly false impressions of efficacy.

A review of the instant specification shows just a single long example wherein a T cell response to a single insulin B chain peptide (amino acids 9-23) is inhibited in the experimental NOD mouse model of IDDM. First note that the instant claims are drawn to the use of GAD, not insulin, for the suspending, preventing or delaying the onset of IDDM. Thus, the specification offers *no* data in support of the claimed method. Interestingly, the specification discloses, that even regarding the use of an insulin peptide for the suspending, preventing or delaying the onset of IDDM, *the method of the instant claims cannot function as claimed*, (emphasis added). For example, at page 28 of the specification, it is disclosed that, "Soluble Ig-INSβ displayed dose dependent delay of diabetes when given at either stage [pre or post IAA conversion]. However, aggregated Ig-INSβ, which induced IL-10 and TGFβ-producing T cells, thus involving sustained endogenous IL-10, was protective against diabetes when given before development of insulitis *but had no effect in predisposed mice positive for IAA*", emphasis added. Further, Examples 7 and 9 teach that neither soluble nor aggregated Ig-INSβ can actually prevent IDDM, but rather can only delay onset under specific conditions.

Additionally, Applicant's subsequent work demonstrates that the method of the instant claims would not be expected to function as claimed. See for example Legge et al. (1998). Therein the authors teach that APLs function as, "T cell

antagonists, partial agonists, or super agonists" (page 106). The authors go on to teach that PLP-LR stimulated PLP-1 specific T cells (paragraph spanning page 109 and 110), i.e., the T cells that would be pathogenic in an MS patient. Given that *no* experiments have been performed employing GAD peptides and derivatives thereof, it is just as likely that the method of the instant claims would actually exacerbate disease as treat or prevent it.

A set forth in *Rasmusson v. SmithKline Beecham Corp.*, 75 USPQ2d 1297, 1302 (CAFC 2005), enablement cannot be established unless one skilled in the art "would accept without question" an Applicant's statements regarding an invention, particularly in the absence of evidence regarding the effect of a claimed invention. Specifically:

> "As we have explained, we have required a greater measure of proof, and for good reason. If mere plausibility were the test for enablement under section 112, applicants could obtain patent rights to "inventions" consisting of little more than respectable guesses as to the likelihood of their success. When one of the guesses later proved true, the "inventor" would be rewarded the spoils instead of the party who demonstrated that the method actually worked. That scenario is not consistent with the statutory requirement that the inventor enable an invention rather than merely proposing an unproved hypothesis."

Thus, in view of the quantity of experimentation necessary, the lack of sufficient guidance in the specification, the lack of sufficient working examples, i.e., the specification discloses **no** data regarding the treatment or prevention of IDDM employing GAD peptides, and the unpredictability of the art, it would take undue trials and errors to practice the claimed invention.

Applicant's arguments, filed 5/20/11, have been fully considered but are not found persuasive. Applicant alleges, "the PTO has not provided any credible evidence showing that one of ordinary skill in the art would ***reasonably doubt*** the asserted utility of the claimed invention and has therefore not met its initial burden" (emphasis by Applicant).

Applicant is advised that no rejection for lack of utility has been made. The invention would most certainly have utility if it functioned as claimed in humans. Type 1 diabetes is a growing scourge on the human population; its prevention would most certainly be one of the greatest achievements in medical history.

Applicant alleges, "the sum total the evidence provided by the Patent Office shows that, using fundamentally different therapeutic agents than presently claimed, tested in diseases other than Type 1 diabetes as presently claimed, some researchers have achieved tolerance results in animal models that have been difficult to reproduce in humans."

Applicant's position is at best, overstated. If the therapeutic agents of the cited references are actually "fundamentally different", then Applicant's method of preventing or delaying type 1 diabetes would likely function through a "fundamentally different" mechanism unknown to biological science. As such, said mechanism would require significant enablement comprising, at the very least, some sort of sound scientific reasoning as to why the ordinarily skilled artisan would have any expectation of success employing Applicant's revolutionary method in the treatment of human disease. Further, it is unclear how Applicant can credibly allege that the diseases of the cited references are "diseases other than Type 1 diabetes" when at least Pozzilli et al. and Skyler et al. specifically report failures in attempted treatments of type 1 diabetes. Applicant is advised that mischaracterizing facts of record does not comprise a persuasive argument.

Citing MPEP 2164.05(a), Applicant again argues that post-filing date references cannot be used in an enablement rejection. Applicant cites *In re Wright* (cited in MPEP 2164.05(a)) and opines on the holdings of the court.

Interestingly, what Applicant fails to point out is that, similar to fact pattern in the instant application, the examiner in the Wright application cited a post-filing reference (Matthews et al.) showing that even some years post-filing the treatment was ineffective in preventing disease in animal models, that animal models were "likely to be imperfect", and that testing in humans was "necessary to determine safety, immunogenicity, and efficacy." The court itself further found relevant the fact that even years after the Wright invention no one had yet developed a "generally successful AIDS virus vaccine". The court also found relevant the fact that the Examiner noted that the scientific community was having difficulty developing an AIDS vaccine years after the filing of the Wright application, "to illustrate that the art is not even today [10 years later] as predictable as Wright has suggested that it was back in 1983." This fact is particularly relevant to the method of the instant claims wherein some 9+ years after the priority date of the instant application no antigen-specific treatments, much less preventions, for type 1 diabetes are known. More specifically, antigen-specific treatments have been tried in humans and failed, see Pozzilli et al. and Skyler et al.

Applicant dismisses the teachings of the Marketletter, Pozzilli et al., Dong et al., Legge et al, and Goodnow.

Applicant's dismissal is noted. The references still serve, however, to teach the unpredictability of establishing immune tolerance in humans. And again, if Applicant's method does not function through the establishment of immune tolerance, then said method would be truly revolutionary in the biological sciences and requiring of more enablement than is set forth in the instant specification which includes no showing of the delaying or preventing of type 1 diabetes employing the GAD construct of the instant claims.

Applicant cites the Inventor's previously submitted 1.132 declaration as showing that the IgGAD2 construct of the instant claims could delay hyperglycemia in the NOD experimental mouse model. Applicant further refers to the NOD mouse model as the "gold standard animal model".

Applicant's position seems to be that while post-filing demonstrations of a lack of enablement are impermissible, post-filing demonstrations of enablement are permissible. Said position would seem to be contradictory. Turning again to *In re Wright*, the court dismissed the post-filing submissions of the Inventor, stating, "all of these developments occurred after the effective filing date of Wright's application and are of no significance regarding what one skilled in the art believed as of that date." Regarding the NOD mouse model comprising the "gold standard animal model", Applicant is reminded that distinguished immunologists such as Gerald Nepom and Matthias von Herrath have referred to the NOD model as a "workhorse" with numerous limitations, see von Herrath and Nepom (2009, of record). They note that just one of several NOD mouse strains even develops diabetes. They further note the ease of treating diabetes in the single NOD strain susceptible to diabetes, i.e., "over 200 perturbations of the immune environment are known that can prevent or reverse disease in NOD mice," treatments that have proven to be ineffective in humans (see Box 1). They conclude that the single strain of NOD mouse is susceptible to diabetes because of, "a rather unique set of genotypic circumstances that is unlikely to exist in a substantial fraction of the human population, if it exists at all" (page 130, column 1).

Applicant argues that treatment in humans is not required.

Applicant's position is noted. It must also be noted, however, that failures of treatments in humans cannot simply be ignored.

5. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

6. Claims 1-5, 7, 13, 15-19, and 22-24, and 26-30 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-7 and 13-16 of U.S. Patent Application No. 11/290,070. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '070 application recite a method comprising treating IDDM with a GAD construct such as would be encompassed by that recited in Claim 1.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

7. Claims 1-5, 7, 13, 15-19, and 22-24, and 26-30 stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over Claims 1-7 and 13-16 of U.S. Patent Application No. 11/425,084. Although the

conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '084 application recite a method comprising treating IDDM with a GAD construct such as would be encompassed by that recited in Claim 1.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Applicant defers a response regarding the remaining double patenting rejections until the finding of allowable claims.

8. Claims 1-5, 7, 13, 15-19, and 22-24, and 26-30 stand rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a new matter written description rejection.

As set forth previously, The specification and the claims as originally filed do not provide support for the invention as now claimed, specifically:

A) A method comprising the administration of an immunoglobulin construct comprising a protein [fragment] (added 11/12/10) represented by SEQ ID NO:4 (Claims 1 and 13).

Applicant cites pages 13, 21, 45, and 26 in support of the claimed method.

A review of the specification reveals that the peptide of SEQ ID NO:4 is found at page 46 of the specification. The specification, however, does not teach the peptides as part of an immunoglobulin construct.

Applicant's arguments, filed 5/20/11, have been fully considered but are not found persuasive. Applicant now cites pages 4, 8, 19, 22-24, 45, and 46 of the specification.

A review of the cites shows that just the cite at pages 45-46 discloses the peptide of SEQ ID NO:4. The generic disclosures of "GAD2" at pages 4, 8, 19, and 22-24 cannot support the claimed method employing a specific chimeric construct, said construct comprising the specific amino acid sequence of SEQ ID NO:4 (TYEIAPVFVLLEYVT).

A review of pages 45 and 46, at the beginning of the Examples section, show that the peptide of SEQ ID NO:4 is disclosed just once, and only in the context of a peptide. It is not disclosed in the context of the claimed method of administering an Ig-GAD65 peptide construct for the delaying or preventing of type 1 diabetes. Indeed, the disclosure of the Examples is limited to the production and administration of a single Ig-insulin peptide (Ig-INSβ) and an Ig-hen egg lysozyme (Ig-HEL) control peptide. Even assuming that the cite supports an Ig-GAD65 construct employing the peptide of SEQ ID NO:4, it does not teach said construct comprising a CDR1, CDR2, and CDR3 as is claimed.

9. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

10. Claims 1, 2, 4, 5, 7, 13, 15-19, 22-24, 26, and 28-30 are rejected under 35 U.S.C. 103(a) as being unpatentable over WO 98/30706 in view of Chao et al. (1999).

As set forth previously, WO 98/30706 teaches the treatment of autoimmune disorders, including IDDM, (see particularly pages 10 and 19) employing an engineered fusion protein, e.g., a humanized IgG_{2b} chimeric protein wherein an autoantigen peptide is inserted into the D segment of a CDR3 loop (see particularly Figure 1, page 13, and Example II).

The method differs from the claimed invention only in that it does not teach the use of the GAD65 SEQ ID NO:4 peptide as the autoantigen employed for the treatment of IDDM.

Chao et al. teach that the GAD65 peptide of SEQ ID NO:4 (p206-220) is an immunodominant T cell diabetes antigen in their NOD mouse diabetes model (see particularly page 9300, Results).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to perform the method of WO 98/30706 for the treatment of IDDM in a subject such as a mouse model of diabetes employing the p206-220 T cell autoantigen of Chao et al. One of ordinary skill in the art at the time the invention was made would have had reason to select the GAD65 p206-220 peptide as the autoantigen of choice for use in the claimed method because Chao et al. teaches that it was the most immunodominant diabetes T cell antigen in their model. Regarding the timing of administration of the Ig-fusion protein set

forth in claims such as 3, 16, 17, etc., said timing would comprise only routine optimization which would fall well within the purview of one of skill in the art at the time of the invention.

Applicant's arguments, filed 5/20/11, have been fully considered but are not found persuasive. Applicant again argues that an obviousness rejection that relies on an alleged teaching, suggestion or motivation *must* be articulated in a *Graham v. Deere* format.

Again, Applicant is simply in error, the *Graham v. Deere* format is just one of many that may be used in a finding of obviousness. It is the substance of a rejection, not the format that is critical.

Applicant again argues a lack of expectation of success.

Applicant's argument seems puzzling given the fact that as of the effective filing date of the instant application the record contains no evidence that Applicant had performed the method of the instant claims, even in their limited animal model. And it is unclear how Applicant can convincingly ignore the Inventor's own teaching (in WO 98/30706) that the Ig chimeric construct can be employed in the treatment of various T cell mediated disorders, including insulin dependent (type 1) diabetes, by simply changing the antigen (pages 10 and 19).

Applicant argues unexpected results.

Applicant is advised that a persuasive claim of unexpected results first requires that the unexpected results be commensurate in scope with the invention as claimed. Such is clearly not the case here. Second, evidence of unexpected results generally takes the form of a direct comparison of the claimed invention with the closest prior art which is commensurate in scope with the claims. In this instance it is noted that no comparison with any other treatment has been performed. Accordingly, Applicant's allegations of unexpected results is not persuasive.

Applicant argues that the prior art does not teach the step of administering the claimed therapy after autoantibody seroconversion.

After autoantibody seroconversion would be the most obvious time to administer treatment of the instant claims. The ordinarily skilled artisan would have viewed this timeframe as the time at which the onset of actual disease could be delayed or prevented in a subject now proven likely to develop disease (because of the seroconversion).

11. No claim is allowed.

12. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Dr. Gerald Ewoldt whose telephone number is (571) 272-0843. The examiner can normally be reached Monday through Thursday from 7:30 am to 5:30 pm. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dr. Ram Shukla, can be reached on (571) 272-0735.

14. **Please Note:** Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197

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EXHIBIT B

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(54) Title: COMPOUNDS, COMPOSITIONS AND METHODS FOR THE ENDOCYTIC PRESENTATION OF IMMUNOSUPPRESSIVE FACTORS

(57) Abstract

Immunomodulating agents comprising at least one Fc receptor ligand and at least one immunosuppressive factor are provided as are methods for their manufacture and use. The immunomodulating agents may be in the form of polypeptides or chimeric antibodies and preferably incorporate an immunosuppressive factor comprising a T cell receptor antagonist. The compounds and compositions of the invention may be used to selectively suppress the immune system to treat symptoms associated with immune disorders such as allergies, transplanted tissue rejection and autoimmune disorders including lupus, rheumatoid arthritis and multiple sclerosis.

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COMPOUNDS, COMPOSITIONS AND METHODS FOR THE ENDOCYTIC PRESENTATION OF IMMUNOSUPPRESSIVE FACTORS

Field of the Invention

The present invention generally relates to compounds, compositions and methods for the effective endocytic presentation of immunosuppressive factors. More particularly, the present invention is directed to compounds, methods and compositions comprising immunosuppressive factors that are useful for the treatment of various disorders including, but not limited to, autoimmune disorders. In preferred embodiments the immunosuppressive factors may be T cell receptor antagonists or agonists. Other embodiments of the invention provide for the induction of tolerance in neonates or infants.

Background of the Invention

Vertebrates possess the ability to mount an immune response as a defense against pathogens from the environment as well as against aberrant cells, such as tumor cells, which develop internally. The immune response is the result of complex interactions between a variety of cells and factors, but generally comprises two main facets. One is a cellular component, in which specialized cells directly attack an offending agent (bearing an antigen) while the other is a humoral component, in which antibody molecules bind specifically to the antigen and aid in its elimination. Acting in concert, the individual elements are quite effective in limiting the initial onslaught of invading pathogens and eliminating them from the host.

The primary cells involved in providing an immune response are lymphocytes which generally comprise two principal classes. The first of these, designated B cells or B lymphocytes, are typically generated in bone marrow and are, among other duties, responsible for producing and secreting antibodies. B cell antibody products tend to react directly with foreign antigens and neutralize them or activate other components of the immune systems which then eliminate them. In particular, opsonizing antibodies bind to extracellular foreign agents thereby rendering them susceptible to phagocytosis and subsequent intracellular killing. On the other hand T cells or T lymphocytes, which generally develop or mature in the thymus, are responsible for mediating the cellular immune response. These cells do not recognize whole antigens but, instead, respond to short peptide fragments thereof bound to specialized proteins which appear on the surface of the surface of a target cell. More particularly, it appears that proteins produced within the cell, or taken up by the cell from the extracellular milieu, are continually degraded to peptides by normal metabolic pathways. The resulting short fragments associate with intracellular major histocompatibility complex (MHC) molecules and the MHC-peptide complexes are transported to the surface of the cell for recognition by T cells. Thus, the cellular immune system is constantly monitoring a full spectrum of proteins produced or ingested by the cells and is posed to eliminate any cells presenting foreign antigens or tumor antigens; i.e. virus infected cells or cancer cells.

The general structure of immunoglobulin G (IgG), the most common of mammalian antibodies, is shown schematically in Figure 1. As illustrated, IgG is a tetrameric protein complex comprising two identical heavy (H)

chains and two identical immunoglobulin light (L) chains. These chains are joined together by disulfide bonds to form the Y-shaped antibody complex. In solution however, the molecule takes on a more globular shape and readily bind to foreign antigens present in biological fluids.

Amino acid sequence analysis of immunoglobulins has led to the definition of specific regions with various functional activities within the chains. Each light chain and each heavy chain has a variable region (V_L and V_H respectively) defined within the first 110 amino terminal residues. Three dimensional pairing of the V_L and V_H regions constitute the antigen-recognition portion or "antigen combining site" ("ACS") of immunoglobulin molecule. Because of the tetrameric nature of immunoglobulins, there are two identical antigen combining sites per molecule. The variable domains of these chains are highly heterogeneous in sequence and provide the diversity for antigen combining sites to be highly specific for a large variety of antigenic structures. The heterogeneity of the variable domains is not evenly distributed throughout the variable regions, but is located in three segments, called complementarity determining regions ("CDRs") designated CDR 1, CDR 2 and CDR 3. For further information regarding these structures see Watson et al., 1987, *Molecular Biology of the Gene*, Fourth Edition, Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA incorporated herein by reference.

Each of the heavy chains also includes a constant region defining a particular isotype and assigns the immunoglobulin to one of the immunoglobulin classes and subclasses. The constant region contains units called domains (i.e. C_{H1}, C_{H2}, etc.) which do not vary significantly among antibodies of a single class. The constant region does not participate in antigen binding, but can be associated with a number of biological activities known as "effector functions", such as binding to Fc receptors on cell surfaces of antigen presenting cells (APC's) as well as binding to complement proteins. Antigen presenting cells such as dendritic cells and macrophages are, among other features, generally distinguished by the presence of an Fc receptor. Consequently, if an antibody is bound to a pathogen, it can then link to a phagocyte via the Fc portion. This allows the pathogen to be ingested and destroyed by the phagocyte, a process known as opsonization. Moreover, as will be discussed in more detail below, various pathogenic antigens may be processed and displayed by the APC to further stimulate an immune response.

Unlike the heavy chains, the light chains have a single constant domain (C_L). A light chain pairs with a heavy chain through a disulfide bond which attaches heavy constant region C_{H1} to C_L. In addition, the heavy chains have a hinge region separating constant regions C_{H1} and C_{H2} from the remainder of the molecule. It is this hinge region that is largely responsible for the flexibility of the tetramer. The two heavy chains of the molecule pair together through disulfide bonds at the junction between the hinge region and C_{H2}.

In order to provide such an extensive repertoire, immunoglobulin genes have evolved so as permit the production of vast numbers of different immunoglobulin proteins from a finite number of genes i.e. inherent polymorphism. Due to inherent polymorphism, mammals are able to produce antibodies to a seemingly infinite variety of antigens. For a review of immunoglobulin genetics and protein structure see Lewin, "Genes III", John Wiley and Sons, N.Y. (1987) and Benjamini and Leskowitz, 1988, Immunology, Alan R. Liss, Inc., New York which is incorporated herein by reference.

In the past few years antibodies have become extremely important in diagnostic and therapeutic applications due to their diversity and specificity. Increasingly, molecular biology techniques have been used to expand the variety and availability of antibodies for scientific applications. For instance, a single antibody producing B cell can be immortalized by fusion with a tumor cell and expanded to provide an *in vitro* source of antibodies of a single specificity known as a "monoclonal antibody" (mAb). Such an immortal B cell line is termed a "hybridoma."

Until recently, the source of most mAb has been murine (mouse) hybridomas cultured *in vitro*. That is, a mouse was typically injected with a selected antigen or immunogen. Subsequently, the animal was sacrificed and cells removed from its spleen were fused with immortal myeloma cells. Although they have been used extensively in diagnostic procedures, murine mAb have not proven to be well suited for therapeutic applications in most mammals including humans. In part, this is due to the fact that murine antibodies are recognized as foreign by other mammalian species and elicit an immune response which may itself cause illness or undesirable side effects.

To overcome at least some of the problems of immune responses generated by foreign mAb and the lack of suitable human mAb, genetic engineering has been used to construct humanized chimeric immunoglobulin molecules which contain the antigen binding complementarity determining regions of the murine antibodies but in which the remainder of the molecule is composed of human antibody sequences which are not recognized as foreign. Such antibodies have been used to treat tumors as the mouse variable region recognizes the tumor antigen and the humanized portion of the molecule is able to mediate an immune response without being rapidly eliminated by the body. See, for example, Jones et al., *Nature*, 321:522-525 (1986) which is incorporated herein by reference.

Other uses of such antibodies are detailed in co-pending U.S.S.N. 08/363,276 and PCT Publication No. WO 94/14847 which are also incorporated herein by reference. In these cases epitopes of foreign antigens such as viral or bacterial epitopes are grafted onto the hypervariable region of an immunoglobulin to induce a response. That is, the engineered antibodies are used as a vaccine to provoke an immune response and confer long term immunogenic memory thereby allowing the subject to fight off subsequent infections.

These and more traditional vaccines are effective in that they stimulate both prongs of the immune system. Despite the intricacies associated with the humoral component of the immune response, it would not, in and of itself, be capable of effectively protecting an animal from the myriad pathogenic assaults to which it is subject each day. Rather, it is only the presence of a highly evolved cellular response that allows higher organisms to survive and proliferate.

As indicated above, T lymphocytes or T cells, which arise from precursors in the bone marrow, are central players in the immune response against invading viruses and other microbes. The progenitor stem cells migrate to the thymus where, as so-called thymocytes, they become specialized. In particular, they begin to display the receptor molecules that later enable mature T cells to detect infection. To be beneficial, T cells must be able to attach through their receptors to microbial antigens (protein markers signaling an invader's presence). At the same time, they should be blind to substances made by the body as self-reactive T cells can destroy normal tissues. Typically, only those thymocytes that make useful receptors will mature fully and enter the bloodstream to patrol the body.

Others that would be ineffectual or would attack the body's own tissue are, in healthy individuals, eliminated through apoptosis prior to leaving the thymus.

Mature T cells that finally enter the circulation, either as cytolytic T lymphocytes or T helper cells, remain at rest unless they encounter antigens that their receptors can recognize. Upon encountering the specific antigens for which the lymphocytes have affinity, they proliferate and perform effector functions, the result of which is elimination of the foreign antigens.

T cells have been classified into several subpopulations based on the different tasks they perform. These subpopulations include helper T cells (T_h), which are required for promoting or enhancing T and B cell responses; cytotoxic (or cytolytic) T lymphocytes (CTL), which directly kill their target cells by cell lysis; and suppressor T cells (T_S) which down-regulate the immune response. In each case the T cells recognize antigens, but only when presented on the surface of a cell by a specialized protein complex attached to the surface of antigen presenting cells. More particularly, T cells use a specific receptor, termed the T cell antigen receptor (TCR), which is a transmembrane protein complex capable of recognizing an antigen in association with the group of proteins collectively termed the major histocompatibility complex (MHC). Thousands of identical TCR's are expressed on each cell. The TCR is related, both in function and structure, to the surface antibody (non-secreted) which B cells use as their antigen receptors. Further, different subpopulations of T cells also express a variety of cell surface proteins, some of which are termed "marker proteins" because they are characteristic of particular subpopulations. For example, most T_h cells express the cell surface CD4 protein, whereas most CTL and T_S cells express the cell surface CD8 protein. These surface proteins are important in the initiation and maintenance of immune responses which depend on the recognition of, and interactions between, particular proteins or protein complexes on the surface of APCs.

For some time it has been known that the major histocompatibility complex or MHC actually comprises a series of glycosylated proteins comprising distinct quaternary structures. Generally, the structures are of two types: class I MHC which displays peptides from proteins made inside the cell (such as proteins produced subsequent to viral replication), and class II MHC, which generally displays peptides from proteins that have entered the cell from the outside (soluble antigens such as bacterial toxins). Recognition of various antigens is assured by inherited polymorphism which continuously provides a diverse pool of MHC molecules capable of binding any microbial peptides that may arise. Essentially, all nucleated cells produce and express class I MHC which may exhibit naturally occurring peptides, tumor associated peptides or peptides produced by a viral invader. Conversely, only a few specialized lymphoid cells, those generally known as antigen presenting cells, produce and express class II MHC proteins. Regardless of the cell type, both classes of MHC carry peptides to the cell surface and present them to resting T lymphocytes. Ordinarily T_h cells recognize class II MHC-antigen complexes while CTL's tend to recognize class I MHC-antigen complexes.

When a resting T cell bearing the appropriate TCR encounters the APC displaying the peptide on its surface, the TCR binds to the peptide-MHC complex. More particularly, hundreds of TCR's bind to numerous peptide-MHC complexes. When enough TCRs are contacted, the cumulative effect activates the T cell. Receptors on T cells that are responsible for the specific recognition of, and response to, the MHC-antigen complex are composed of a complex

of several integral plasma membrane proteins. As with the MHC complex previously discussed, a diverse pool of TCR's is assured by inherent polymorphism leading to somatic rearrangement. It should be emphasized that, while the pool of TCR's may be diverse, each individual T cell only expresses a single specific TCR. However, each T cell typically exhibits thousands of copies of this receptor, specific for only one peptide, on the surface of each cell. In addition, several other types of membrane associated proteins are involved with T cell binding and activation.

Activation of the T cell entails the generation of a series of chemical signals (primarily cytokines) that result in the cell taking direct action or stimulating other cells of the immune system to act. In the case of class I MHC-antigen activation, CTL's proliferate and act to destroy infected cells presenting the same antigen. Killing an infected cell deprives a virus of life support and makes it accessible to antibodies, which finally eliminate it. In contrast, activation of T_h cells by class II MHC-antigen complexes does not destroy the antigen presenting cell (which is part of the host's defense system) but rather stimulates the T_h cell to proliferate and generate signals (again primarily cytokines) that affect various cells. Among other consequences, the signaling leads to B cell stimulation, macrophage activation, CTL differentiation and promotion of inflammation. This concerted response is relatively specific and is directed to foreign elements bearing the peptide presented by the class II MHC system.

When operating properly the immune response is surprisingly effective at eliminating microscopic pathogens and, to a lesser extent, neoplastic cells. In general, the complicated mechanisms for self-recognition are very efficient and allow a strong response to be directed exclusively at foreign antigens. Unfortunately, the immune system occasionally malfunctions and turns against the cells of the host thereby provoking an autoimmune response. Typically, autoimmunity is held to occur when the antigen receptors on immune cells recognize specific antigens on healthy cells and cause the cells bearing those particular substances to die. In many cases, autoimmune reactions are self-limited in that they disappear when the antigens that set them off are cleared away. However, in some instances the autoreactive lymphocytes survive longer than they should and continue to induce apoptosis or otherwise eliminate normal cells. Some evidence in animals and humans indicates that extended survival of autoreactive cells is implicated in at least two chronic autoimmune disorders, systemic lupus erythematosus and rheumatoid arthritis.

Other mechanisms of action are also thought to contribute to the development of various autoimmune disorders. For example, over the last few years it has become clear that the avidity of T cell-APC interactions dictates thymic learning and tolerance to self antigens. Accordingly, high avidity interactions lead to elimination of the T cell whereas low avidity interactions allow for maturation and exit from the thymus. Although this mechanism is effective in purging the immune system of autoreactivity, T cell precursors endowed with self reactivity could still be generated and migrate to the periphery if the autoantigen is sequestered and does not achieve effective levels of thymic presentation, is subjected to thymic crypticity, or is poorly presented. Moreover, superantigens capable of reacting with particular T cell receptors and events that could stimulate antigen mimicry, epitope spreading or peripheral loosening in peptide crypticity may trigger activation of those self-reactive T cells and cause antigen exposure. In any case, continuous supply of autoantigen and abundant generation of T cell receptor ligands (peptide-MHC complexes) are a likely mechanism of T cell aggressivity. Examples of such a spontaneous break in self-

tolerance include multiple sclerosis (MS), rheumatoid arthritis (possibly more than one mechanism) and type I diabetes all of which are thought to be T cell mediated autoimmune diseases.

Regardless of which mechanism is responsible for the corruption of the immune system, the results can be devastating to the individual. For example, multiple sclerosis is a chronic, inflammatory disorder that affects approximately 250,000 individuals in the United States. The inflammatory process occurs primarily within the white matter of the central nervous system and is mediated by T cells, B cells and macrophages which are responsible for the demyelination of the axons. Although the clinical course can be quite variable, the most common form is manifested by relapsing neurological deficits including paralysis, sensory deficits and visual problems.

Once immune cells have spread to the white matter of the central nervous system, the immune response is targeted to several different antigens on myelin. For example, there is a critical antibody response directed to myelin that activates the complement cascade with membrane attack complexes appearing in the spinal fluid. Further, T cells are targeted to certain key portions of various myelin antigens such as those presented on myelin basic protein (MBP) and proteolipid protein (PLP). The T cells in turn produce cytokines which then influence macrophages to attack the myelin and phagocytose large chunks of the myelin sheath. The concerted attack leads to areas of demyelination impairing salatory conduction along the axon and producing and the pathophysiologic defect. Multiple immune responses to several components of a supramolecular structure, like the myelin sheath in multiple sclerosis or the pyruvate dehydrogenase complex in primary biliary cirrhosis, are common in individuals with autoimmune diseases involving discrete organs.

Treatments for autoimmune diseases have met with varying levels of success. For example, it is often possible to correct organ-specific autoimmune disease through metabolic control. Where function is lost and cannot be restored, mechanical substitutes or tissue grafts may be appropriate. However, no effective treatments exist for several of the most disabling disorders including MS. While a number of compounds, including corticosterioids and modified beta interferon, can reduce some symptoms of MS, they have proven to have serious side effects or otherwise been shown to be less than desirable for long term use. Other avenues of treatment have shown promise but have yet to be shown effective.

In this respect, one promising treatment for MS is described in WO 96/16086, incorporated herein by reference, which discloses the use of peptide analogs of myelin basic protein (MBP). Compositions comprising these analogs are reportedly able to ameliorate symptoms of MS without excessive side effects. Moreover, use of peptide analogs to myelin constitutive proteins were also shown to be effective in treating the symptoms of experimental allergic encephalomyelitis (EAE), an organ specific immune disorder often used in mice as a model for MS. Specifically, reversal of EAE was achieved with a peptide analog derived from proteolipid (PLP) peptide (Kuchroo et al., *J. Immunol.* 153:3326-3336, 1994, incorporated herein by reference). It was shown that when the major TCR contacting residues within the naturally occurring PLP peptide were mutated, the resulting peptide analog bound MHC as well as the natural peptide yet does not activate PLP specific T cells. Instead the PLP analog inhibits *in vitro* activation of the T cells.

While peptide analogs represent an attractive approach to modulate the effector functions of aggressive T cells and ameliorate autoimmune diseases, several problems limit their effectiveness. For instance, only a few MHC-peptide complexes are available on the surface of a typical APC meaning a single complex may be required to serially trigger about 200 TCRs to activate the T cell. Where the autoantigen is continuously available for normal processing and presentation by the MHC system, it appears that very few surface MHC complexes would be available to bind the peptide analog. Further, as free peptides typically have very short half-lives, they are not readily incorporated and processed by the MHC-antigen presenting system, little will be naturally expressed on the APC. Due to the inefficient presentation, direct inhibition of the thousands of TCR's on each T cell likely require prohibitively high intracellular levels of free peptide. The turnover of cell surface MHC molecules also contributes to the short stay of complexes formed at the extracellular milieu (i.e. MHC class II molecules have been in the cell surface for some time before binding the extracellular peptide) while complexes formed in the endocytic compartment will reside for a normal period of time because they have just been translocated to the cell surface. Finally, as previously alluded to, administration of such synthetic epitopes or analogs is extremely problematic in view of the short half-life of peptides in the mammalian body. Between the short half-lives of the MHC complexes and the administered peptides, effective exposure is too brief to permit the induction of a satisfactory immune response further necessitating higher doses.

Accordingly, it is a general object of the present invention to provide methods and associated compositions for effectively modifying the immune system of a vertebrate to treat an immune disorder.

It is another object of the present invention to provide methods and compositions for the effective presentation of T cell receptor antagonists or agonists to modulate the cellular immune response in a subject in need thereof.

It is yet a further object of the present invention to provide methods and compositions for the treatment and amelioration of various immune disorders.

It is yet another object of the present invention to provide methods and compositions for the induction of T cell tolerance in neonates or infants.

It is still another object of the present invention to provide for the relief of pathological symptoms associated with autoimmune disorders including multiple sclerosis.

Summary of the Invention

These and other objectives are accomplished by the methods and associated compounds and compositions of the present invention which, in a broad aspect, provides for an Fc receptor mediated, endocytic delivery system. In selected embodiments the invention provides for the effective presentation of immunosuppressive factors which, in preferred embodiments may comprise T cell receptor antagonists or agonists. More particularly, the present invention provides methods, compounds and compositions to present immunosuppressive factors for the selective modification of an immune response in a vertebrate. In particularly preferred embodiments, the invention provides for Fc receptor mediated endocytic presentation of a selected T cell receptor antagonist or agonist to modulate an

immune response mounted against a specific antigen. As will be appreciated by those skilled in the art, the disclosed methods and compositions may be used to treat any physiological disorder related to the immune response of a vertebrate. For example, this ability to suppress selected components of the immune system may allow, among other things, for the treatment of autoimmune diseases, facilitation of tissue or organ transplants and the mitigation of symptoms produced by allergens. Moreover, the present invention further provides for the induction of tolerance in neonates and infants with regard to autoantigens.

In preferred aspects of the invention, the endocytic presentation of the selected immunosuppressive factor is facilitated through the use of an immunomodulating agent that is able to bind to the Fc receptor (FcR) of antigen presenting cells. Typically, the immunomodulating agent will comprise at least one immunosuppressive factor associated with at least one ligand capable of binding to a Fc receptor. Upon binding to the antigen presenting cell (APC) the immunomodulating agent will be internalized and processed by the APC's natural endocytic pathway. Preferably, the internalized immunosuppressive factor, which can be a T cell receptor antagonist or agonist, will then be associated with the newly synthesized endogenous MHC class II structures and presented at the surface of the APC. Those skilled in the art will appreciate that the immunosuppressive factors, while complexing with T cell receptors when bound to MHC class II structures, will not promote activation of the T cell. It will further be appreciated that hundreds of TCR's on each T cell must be triggered in order to activate the cell. Accordingly, efficient presentation of an appropriate TCR antagonist or agonist can prevent a previously primed T cell (i.e. one sensitized to a particular autoantigen) from activating and triggering an immune response despite competitive presentation of the naturally occurring autoantigen.

In a broad sense, the immunomodulating agents of the present invention may comprise any ligand (FcR ligand) that is capable of binding to, and being internalized by, the Fc receptor of an antigen presenting cell. That is, the FcR ligand may be any protein, protein fragment, peptide or molecule that effectively binds to a Fc receptor on the surface of any antigen presenting cell. Preferably, the FcR ligand will comprise or mimic at least some portion of a constant region of an immunoglobulin molecule and will not provoke an antigenic response in the subject. In selected aspects of the invention, the FcR ligand will comprise part or all of a constant region from an IgG molecule. Particularly preferred embodiments will employ FcR ligands comprising the entire constant region of a selected immunoglobulin molecule from the species to be treated. Of course, it will also be appreciated that binding to the Fc receptor may also be effected by ligands that comprise small fragments of a single constant region domains or non amino acid based molecular entities. In any case, the FcR ligand may be derived using modern pharmaceutical techniques such as directed evolution, combinatorial chemistry or rational drug design.

As previously alluded to, the compounds of the present invention further comprise an immunosuppressive factor associated with the FcR ligand to provide an immunomodulating agent. For the purposes of the instant invention the immunosuppressive factor can be any molecular entity that is capable of being processed by an APC and presented in association with class II MHC molecules on the cell surface. In particularly preferred embodiments the immunosuppressive factor comprises all or part of a T cell antagonist. For the purposes of this disclosure the term "antagonist" shall, in accordance with its normal meaning, comprise any substance that interferes with the

physiological action of another by combining with, and blocking, its receptor. More particularly, TCR antagonists are molecular entities that, in combination with class II MHC molecules, are capable of non-reactively associating with a T cell receptor and preventing that receptor from binding to its normal activating antigen ligand (i.e. an MHC-peptide agonist). Preferably, the TCR antagonist comprises a peptide or protein fragment that is an analog of the normal activating antigen agonist. In particularly preferred embodiments the TCR antagonist is an analog of a T cell epitope.

In other preferred embodiments the immunosuppressive factor may comprise a T cell agonist that forms a MHC complex which does not activate the primed TCR upon binding. For the purposes of the present disclosure, the term "agonist" shall be used in accordance with its commonly accepted biochemical meaning. In this regard it will be appreciated that, while the T cell agonist may be any molecule that provides the desired immunogenic result, the selected agonist will preferably comprise a peptide or protein fragment. Moreover, those skilled in the art will appreciate that immunomodulating agents comprising one or more T cell receptor agonists may be combined with immunomodulating agents comprising one or more T cell receptor antagonists to provide pharmaceutical formulations that may be used to selectively attenuate a patient's immune response.

In the disclosed compounds and associated methods, the FcR ligand is associated with the immunosuppressive factor to form an immunomodulating agent so that both are internalized by the APC at substantially the same time. This association may be in the form of two or more molecules bound to each other as with an antibody-antigen complex or, in preferred embodiments, may comprise the formation of a single chimeric molecule incorporating both the immunosuppressive factor (i.e. a TCR antagonist or agonist) and FcR ligand. For example, a selected TCR antagonist could be chemically linked to an FcR ligand region produced by proteolytic techniques (i.e. an Fc fragment). Other embodiments may comprise a normal immunoglobulin comprising an FcR ligand sterically bound to an antagonistic or agonistic peptide. Particularly preferred embodiments of the invention comprise chimeric immunoglobulins produced through genetic engineering techniques. In these compounds the FcR ligand (and usually the majority of the molecule) comprises one or more immunoglobulin constant regions while one or more of the variable regions is engineered to express a desired peptide TCR antagonist or TCR agonist. Those skilled in the art will appreciate that any combination of the aforementioned immunomodulating agents may be associated to form compositions of the present invention as can similar immunomodulating agents comprising different immunosuppressive factors. Moreover, as previously alluded to, mixtures or "cocktails" of various immunomodulating agents are specifically contemplated as falling within the scope of the present invention.

The disclosed compositions may be formulated using conventional pharmaceutical techniques and carriers and may be administered through the usual routes. However, the use of FcR mediated uptake of the immunomodulating agent avoids many of the problems associated with prior art compositions. More specifically, the methods of the present invention overcome many of the limitations associated with the administration of free peptide antagonists as disclosed in the prior art. Accordingly, efficient endocytic presentation of an immunosuppressive factor such as a TCR antagonist can generate significant levels of MHC-antagonist ligands to oppose abundant MHC-autoantigenic complexes that are generated in spontaneous immune disorders involving the continuous presentation

of an autoreactive antigen. As such, the invention may be used to treat any immune disorder that responds to the presentation of immunosuppressive factors. This is particularly true of T cell mediated autoimmune disorders including, for example, multiple sclerosis, lupis, rheumatoid arthritis, scleroderma, insulin-dependent diabetes and ulcerative colitis. In a like manner, the present invention can be used to selectively downregulate the immune system with respect to continuously presented agonists such as allergens. Further, the compounds and associated compositions of the present invention may be used to selectively suppress various components of the immune system to reduce the likelihood of tissue or organ rejection following transplant.

In addition, it has been surprisingly found that the compounds, compositions, and methods of the present invention may be used to induce tolerance to various autoantigens in neonates and infants. More particularly, the present invention further provides compositions and methods for conferring resistance in neonate or infant mammals to the induction of an autoimmune disease during adult life. In accordance with the teachings herein this neonatal tolerance is characterized by a lymph node deviation and unusual gamma interferon-mediated splenic anergy upon challenge with the appropriate autoantigen. Further, in preferred embodiments the present invention may provide for the induction of the desired neonatal tolerance without the use of adjuvants (such as incomplete Freund's adjuvant).

Other objects, features and advantages of the present invention will be apparent to those skilled in the art from a consideration of the following detailed description of preferred exemplary embodiments thereof taken in conjunction with the figures which will first be described briefly.

Brief Description of the Drawings

Figs. 1A and 1B are schematic representations of chimeric immunoglobulin G (IgG) molecules illustrating the general features thereof and the inclusion of foreign peptides within the CDR 3 loop of the heavy chain variable region wherein Fig. 1A (Ig-PLP1) shows the insertion of a naturally occurring peptide PLP1 (agonist) derived from proteolipid protein while Fig. 1B (Ig-PLP-LR) illustrates an immunomodulating agent comprising the inclusion of a peptide analog (antagonist) to PLP1 termed PLP-LR;

Figs. 2A and 2B are graphical representations illustrating the capture of chimeric antibodies Ig-PLP1 and Ig-PLP-LR, which correspond to those shown in Figs. 1A and 1B respectively, using antibodies directed to the corresponding free peptides wherein Fig. 2A shows capture levels by antibodies directed to PLP1 and Fig. 2B shows capture levels by antibodies directed to PLP-LR with Ig-W, a wild type antibody, acting as a negative control;

Figs. 3A and 3B are graphs illustrating the presentation of Ig-PLP1 and Ig-PLP-LR (as well as positive and negative controls) to PLP1-specific T cell hybridomas 4E3 (Fig. 3A) and 5B6 (Fig. 3B) to determine the relative T cell activation potentials of the chimeric immunoglobulins as measured by IL-2 production;

Fig. 4 is a graphical representation illustrating the relative effectiveness of presenting PLP1 using the chimeric antibodies of the present invention (Ig-PLP1) versus the free peptide PLP1 or the native proteolipid protein (PLP) as measured by levels of IL-2 production following incubation with splenic SJL antigen presenting cells and PLP1 specific 4E3 T cell hybridoma;

Figs. 5A, 5B and 5C are graphical comparisons showing Ig-PLP-LR antagonism of PLP1 (5A), Ig-PLP1 (5B) and PLP (5C) mediated T cell activation as measured by IL-2 production by T cell hybridoma 4E3 in the presence of SJL splenic APCs that were previously incubated with the respective agonist and various levels of Ig-PLP-LR or controls;

Fig. 6 is a graph showing the relative antagonism of Ig-PLP2, Ig-PLP-LR and Ig-W as measured by the production of IL-2 by T cell hybridoma HT-2 in the presence of SJL splenic APCs that were previously incubated with native proteolipid protein in combination one of the aforementioned immunoglobulins;

Figs. 7A and 7B are graphs demonstrating the *in vivo* presentation of PLP1 following inoculation with Ig-PLP1 as measured by ^{3}H-thymidine incorporation by cells from the lymph node (7A) or the spleen (7B) wherein the illustrated values represent the ability of cells harvested from individual mice to generate a T cell response as measured by ^{3}H-thymidine incorporation when exposed to agonist PLP1 or the control peptide PLP2;

Figs. 8A and 8B are graphical representations showing the ability of Ig-PLP-LR to reduce the immune response to PLP1 peptide when co-administered with Ig-PLP1 as measured in murine cells from the lymph node (8A) or the spleen (8B) wherein the illustrated values represent the ability of cells harvested from individual mice to generate a T cell response as measured by ^{3}H-thymidine incorporation when exposed to PLP1;

Figs. 9A and 9B are graphs demonstrating that mice inoculated with a mixture of Ig-PLP-LR and Ig-PLP1 develop a more vigorous immune response to the peptide analog PLP-LR than peptide PLP1 as measured in cells from the lymph node (9A) or the spleen (9B) wherein the illustrated values represent the ability of cells harvested from individual subjects to generate a T cell response as reflected by ^{3}H-thymidine incorporation when exposed to either PLP1 peptide or the peptide analog PLP-LR.

Figs. 10A-10D are graphical representations of lymph node proliferative responses to immunization with Ig-PLP chimeras with mice individually tested in triplicate wells for each stimulator and where the indicated cpms represent the mean ± SD after deduction of background cpms;

Fig. 11 is a graphical representation of lymph node T cell proliferative response to co-immunization with Ig-PLP1 and Ig-PLPLR with stimulators comprising PPD, 5 μg/ml; PLP 1, PLP-LR, and PLP2 at 15 μg/ml;

Fig. 12 is a graphical representation of splenic proliferative T cells responses of mice immunized with Ig-W, Ig-PLP1, IG-PLP-LR and combinations thereof when stimulated with PLP1 (filled bars) and PLP-LR (hatched bars) in triplicate wells;

Figs. 13A-13C are graphical representations of IL-2 (13A), INFγ (13B), and IL-4 (13C) production by splenic cells of mice immunized with Ig-W, Ig-PLP1, Ig-PLP-LR and combinations thereof;

Figs. 14A-14D graphically illustrate proliferation of antigen experienced T cells from mice immunized with Ig-PLP1 (a and b) or Ig-PLP-LR (c and d) in CFA upon stimulation in vitro with PLPI peptides, PLP-LR peptides and mixtures thereof;

Figs. 15A and 15B are graphical representations of IL-2 production by antigen experienced T cells immunized with Ig-PLP1 (15A) and Ig-PLP-LR (15B) upon in vitro stimulation with PLP1 peptide, PLP-LR peptide or mixtures thereof;

Figs. 16A and 16B graphically illustrate that neonatal mice injected with Ig-PLP1 and Ig-W resist induction of EAE with clinically derived curves shown for all mice (16A) and for surviving mice (16B);

Figs. 17A and 17B graphically show *in vivo* presentation of Ig-PLP1 by neonatal thymic (17A) and splenic (17B) antigen presenting cells following injection with Ig-PLP1 or Ig-W within 24 hours of birth;

Figs. 18A and 18B graphically illustrate lymph (18A) and splenic (18B) proliferative T cell response in mice injected with Ig-PLP1 or Ig-W shortly after birth upon stimulation with free PLP1, PLP2 or a negative control peptide corresponding the encephalitogenic sequence 178-191 of PLP;

Figs. 19A-19C graphically represent lymph node T cell deviation as measured by production of IL-2 (19A), IL-4 (19B), and INFγ (19C) in mice treated with Ig-PLP1 shortly after birth and stimulated with free PLP1 or PLP2;

Figs. 20A-20C graphically represent splenic T cell deviation as measured by production of IL-2 (20A), IL-4 (20B), and INFγ (20C) in mice treated with Ig-PLP1 shortly after birth and stimulated with free PLP1 or PLP2; and

Fig. 21 graphically illustrates cytokine mediated restoration of splenic T cell proliferation in mice injected with Ig-PLP1 shortly after birth, immunized with free PLP1 at seven weeks and stimulated with free PLP1 with the cells grown in control media (NIL) media with IL-12 and media with INFγ with the indicated cpms for each mouse representing the mean ± SD of triplicate wells.

Detailed Description of the Preferred Embodiment

While the present invention may be embodied in many different forms, disclosed herein are specific illustrative embodiments thereof that exemplify the principles of the invention. It should be emphasized that the present invention is not limited to the specific embodiments illustrated.

As previously alluded to, the present invention provides compounds, compositions and methods for selectively modifying the immune response of a vertebrate using an Fc receptor mediated endocytic delivery system. Essentially, any immunomodulating agent that can exploit this form of cellular uptake to downregulate the immune system is held to constitute part of the present invention. Among other forms, the immunomodulating agents of the invention may comprise single polypeptides, antigen-antibody complexes, chimeric antibodies or non-peptide based immunoactive compounds. In preferred embodiments the immunomodulating compounds disclosed herein will comprise at least one FcR ligand and at least one immunosuppressive factor that is capable of downregulating an immune response upon endocytic presentation. Particularly preferred embodiments of the invention comprise an immunomodulating agent wherein the immunosuppressive factor is a T cell receptor antagonist or agonist that is capable of binding with a receptor on the surface of a primed T cell but not capable of generating an immunogenic response. In such embodiments, the presented immunosuppressive factor will effectively compete with selected naturally occurring autoantigens thereby preventing the activation of the corresponding primed T cells and reducing the response generated. This selective suppression of the immune system may, among other indications, be used to treat

symptoms associated with immune disorders, including T cell mediated autoimmune disorders, allergies and tissue rejection in transplant operations.

Accordingly, in one embodiment the present invention comprises an immunomodulating agent for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising at least one Fc receptor ligand and at least one immunosuppressive factor. Preferred embodiments comprise a Fc receptor ligand corresponding to at least a part of an immunoglobulin constant region domain while the immunosuppressive factor corresponds to at least one T cell receptor antagonist. Other preferred embodiments incorporate an immunosuppressive factor comprising a T cell receptor agonist. In particularly preferred embodiments the immunomodulating agent comprises a recombinant polypeptide or a chimeric antibody.

By exploiting FcR mediated uptake of the selected immunomodulating agent the present invention very cleverly uses the body's own metabolic pathways to downregulate harmful immune responses. More specifically, the present invention uses the fact that T cells only recognize and respond to foreign antigens only when attached to the surface of other cells. Selection of the appropriate immunomodulating agent or agents in accordance with the teachings herein provides for the efficient uptake of the administered compound. Following FcR mediated uptake, the natural endocytic pathway of antigen presenting cells provides for the effective presentation of the selected immunosuppressive factor complexed with the MHC class II molecules.

As described above, the two requisite properties that allow a cell to function as an antigen presenting cell for class II MHC-restricted helper T cell lymphocytes are the ability to process endocytosed antigens and the expression of class II MHC gene products. Most cells appear to be able to endocytose and process protein antigens. Accordingly, the determining factor appears to be the expression of class II MHC molecules. In this respect, the best defined antigen presenting cells for helper T lymphocytes comprise mononuclear phagocytes, B lymphocytes, dendritic cells, Langerhans cells of the skin and, in some mammals, endothelial cells. Of course it will be appreciated that different cells may be concentrated in different areas and may be involved in different stages of the T cell mediated immune response. In any case, the term "antigen presenting cell" or "APC" as used herein shall be held to mean any cell capable of inducing a T cell mediated immune response through the processing and surface presentation of an MHC class II-antigen complex. As such, the selected FcR ligand may interact with any of a number of different Fc receptors found on a variety of cell types to promote endocytosis of the immunomodulating agent. By way of example only, selected human Fc receptors that may be employed include the FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA or FcγRIIIB subfamilies.

More generally, in accordance with the present invention those skilled in the art will appreciate that any ligand capable of binding to an FcR complex and initiating endocytosis is compatible with the present invention and may be incorporated in the disclosed immunomodulating agents. Accordingly, FcR ligands may comprise, but are not limited to, peptides, proteins, protein derivatives or small molecular entities that may or may not incorporate amino acids. For example, small molecules derived using modern biochemical techniques such as combinatorial chemistry or rational drug design may be employed as long as they provide for the requisite APC uptake.

While it must be emphasized that any type of compatible molecule may be used, the FcR ligands of the present invention will preferably comprise one or more peptides. More preferably, the FcR ligand will comprise at least a part of a domain of a constant region of an immunoglobulin. In particularly preferred embodiments the FcR ligand will comprise one or more domains derived from a constant region of an immunoglobulin molecule. Those skilled in the art will appreciate that various immunoglobulin isotypes and allotypes may be employed as desired. For example, compatible FcR ligands may be selected from amino acid sequences corresponding to those found in the constant regions of IgG, IgE, IgA or IgM. Among other factors, selection of a particular isotype for use as a FcR ligand may be predicated on biochemical properties such as binding coefficients or low immunoreactivity in the species to be treated. Similarly, the selection of a single domain, fragment thereof or multiple domains may be determined based on biochemical factors or, ultimately, presentation efficiency.

Yet, efficient presentation via the endocytic pathway is typically not enough to selectively downregulate the immune response with regard to a particular antigen. Accordingly, immunomodulating agents of the present invention further comprise an immunosuppressive factor. In accordance with the scope of the present invention the immunosuppressive factor may be any compound that, when endocytically processed and presented on the surface of an APC in conjunction with a MHC class II complex, will downregulate the immune system. As such, immunosuppressive factors may comprise small molecules, peptides, protein fragments, or protein derivatives. In preferred embodiments the immunosuppressive factor acts as an antagonist when presented on the surface of the APC in that it interferes with the binding of a similarly presented agonist to a selected receptor. In particularly preferred embodiments the immunosuppressive factor comprises a T cell receptor antagonist that will associate with a T cell receptor without activating an immune response. Further, other embodiments of the invention comprise immunomodulating agents incorporating T cell receptor agonists that reduce the immune response to the subject autoantigen.

While any functionally compatible molecule may be used as an immunosuppressive factor in accordance with the present invention, those skilled in the art will appreciate that protein fragments or peptides are particularly suitable for use in the disclosed compounds and methods. Such molecules are readily processed by the normal endocytic pathways and are easily presented in concert with the MHC class II molecules on the surface of the antigen presenting cell. Moreover, as the majority of agonist compounds evoking an unwanted immune response are typically protein fragments, T cell receptors are usually most responsive to similar fragments whether they are agonists or antagonists. In particularly preferred embodiments, the immunosuppressive factor will be an analog of a selected peptide or protein fragment that is immunoreactive with a chosen T cell receptor.

"Peptide analogs" or "analogs," as used herein, contain at least one different amino acid in the respective corresponding sequences between the analog and the native protein fragment or peptide. Unless otherwise indicated a named amino acid refers to the L-form. An L-amino acid from the native peptide may be altered to any other one of the 20 L-amino acids commonly found in proteins, any one of the corresponding D-amino acids, rare amino acids, such as 4-hydroxyprofine, and hydroxylysine, or a non-protein amino acid, such as B-alanine and homoserine. Also included with the scope of the present invention are amino acids which have been altered by chemical means such

as methylation (e.g., a-methylvaline), amidation of the C-terminal amino acid by an alkylamine such as ethylamine, ethanolamine, and ethylene diamine, and acylation or methylation of an amino acid side chain function (e.g., acylation of the epsilon amino group of lysine).

Methods for selecting efficient peptide antagonists for treating multiple sclerosis (MS) are provided in PCT Publication No.: WO 96/16086 which has previously been incorporated into the instant application by reference. The disclosed methods may be used in concert with the present invention to provide effective immunosuppressive factors for incorporation in the disclosed immunomodulating agents. For example, using assays detailed below candidate peptide analogs may be screened for their ability to treat MS by an assay measuring competitive binding to MHC, T cell proliferation assays or an assay assessing induction of experimental encephalomyelitis (EAE). Those analogs that inhibit binding of the native autoreactive peptides, do not stimulate proliferation of native peptide reactive cell lines and inhibit the development of EAE (an experimental model for MS) by known autoantigens are useful for therapeutics. Those skilled in the art will appreciate that similar types of assays may be used to screen immunosuppressive factors for other native peptides (i.e. continuously presented autoantigens) and other immune disorders. In particularly preferred embodiments the selected immunosuppressive factors comprise analogs of T cell epitopes.

More generally, immunosuppressive factors may be derived for a number of diseases having a variety of immunoreactive agents without undue experimentation. For example, peptide analog antagonists or agonists may be generated for T cell epitopes on both proteolipid protein or myelin basic protein to treat multiple sclerosis. Similarly, T cell receptor antagonists or agonists may be derived from T cell epitopes of the pyruvate dehydrogenase complex to treat primary biliary cirrhosis. In both cases the derived immunosuppressive factors will be incorporated in a immunomodulating agent as described herein and administered to a patient in need thereof. Effective presentation of the immunosuppressive factor will selectively reduce stimulation of the autoreactive T cells by native peptide thereby relieving the symptoms of the subject immune disorder.

The selected immunosuppressive factor and FcR ligand, together comprising an immunomodulating agent, may be effectively administered in any one of a number of forms. More particularly, as described above, the immunomodulating agents of the present invention may combine any form of the respective elements that are functionally effective in selectively suppressing the immune response. For example, the immunomodulating agent may comprise a recombinant polypeptide or protein produced using modern molecular biology techniques. In such cases the FcR ligand may comprise a fragment of a single immunoglobulin region constant domain or, preferably, the entire constant region. In other embodiments the immunomodulating agent may comprise a sterically bound antibody-antigen complex wherein the antigen comprises a T cell receptor antagonist or agonist. Other preferred embodiments feature an immunomodulating agent comprising a chimeric antibody wherein an immunosuppressive factor is expressed on the Fab fragment. In still other embodiments the immunomodulating agent may comprise two covalently linked molecules which comprise a effective FcR ligand and immunosuppressive factor respectively.

Particularly preferred embodiments of the instant invention will employ recombinant nucleotide constructs to code for immunomodulating agents comprising a single fusion polypeptide. Those skilled in the art will appreciate

that standard genetic engineering technology can provide fusion proteins or chimeras that will comprise at least one FcR ligand and at least one immunosuppressive factor. As used herein the terms "chimera" or "chimeric" will be used in their broadest sense to encompass any polynucleotide or polypeptide comprising sequence fragments from more than one source. For example, a genetically engineered polypeptide incorporating a peptide TCR antagonist and a single Fc domain from an IgG molecule could properly be termed a chimeric or fusion protein. Similarly, a chimeric antibody may comprise a recombinant heavy chains engineered to incorporate a heterologous peptide immunosuppressive factor and a wild type light chains. For the purposes of the present invention, it is not necessary that the disparate regions be derived from different species. That is, a chimeric antibody may comprise human light and heavy chains and an engineered human TCR antagonist expressed in a CDR. Conversely, chimeric immunomodulating agents may comprise FcR ligands and immunosuppressive factors derived from different species such a human and mouse. As such, one aspect of the present invention comprises recombinant polynucleotide molecule encoding a polypeptide wherein said polynucleotide molecule comprises at least one nucleotide sequence corresponding to a Fc receptor ligand and at least one nucleotide sequence corresponding to an immunosuppressive factor. Preferably the immunosuppressive factor will correspond to a T cell receptor antagonist or agonist and the Fc receptor ligand corresponds to at least one constant region domain of an immunoglobulin. In a particularly preferred embodiment the polynucleotide molecule encodes a nucleotide sequence corresponding to an immunoglobulin heavy chain wherein a complementarity determining region has been at least partially deleted and replaced with a nucleotide sequence corresponding to a T cell receptor antagonist or agonist. Compositions comprising mixtures of immunosuppressive factors may also be used effectively in accordance with the teachings herein.

In any case, DNA constructs comprising the desired immunomodulating agents may be expressed in either prokaryotic or eukaryotic cells using techniques well known in the art. See, for example, Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1982 which is incorporated herein by reference. In preferred embodiments the engineered plasmid will be transfected into immortal cell lines which secrete the desired product. As known in the art, such engineered organisms can be modified to produce relatively high levels of the selected immunomodulating agent. Alternatively, the engineered molecules may be expressed in prokaryotic cells such as E. coli. Whatever production source is employed, products may be separated and subsequently formulated into deliverable compositions using common biochemical procedures such as fractionation, chromatography or other purification methodology and conventional formulation techniques.

Accordingly, another aspect of the invention comprises a method for producing an immunomodulating agent for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising the steps of:

a. transforming or transfecting suitable host cells with a recombinant polynucleotide molecule comprising a nucleotide sequence which encodes a polypeptide comprising at least one Fc receptor ligand and at least one immunosuppressive factor;

b. culturing the transformed or transfected host cells under conditions in which said cells express the recombinant polynucleotide molecule to produce said polypeptide wherein the polypeptide comprises at least a part of an immunomodulating agent; and

c. recovering said immunomodulating agent.

Similarly, another aspect of the invention comprises transfected or transformed cells comprising a recombinant polynucleotide molecule encoding a polypeptide wherein the polypeptide comprises at least one Fc receptor ligand and at least one immunosuppressive factor.

In both of the preceding aspects, the immunosuppressive factor is preferably a T cell receptor antagonist or agonist and the Fc receptor ligand preferably comprises at least part of an immunoglobulin constant region domain. More preferably, the immunomodulating agent comprises a poly peptide or chimeric antibody wherein at least one complementarity determining region (CDR) has been replaced with a T cell receptor antagonist or agonist.

It will further be appreciated that the chimeric antibodies, polypeptides and other constructs of the present invention may be administered either alone, or as pharmaceutical composition. Briefly, pharmaceutical compositions of the present invention may comprise one or more of the immunomodulating agents described herein, in combination with one or more pharmaceutically of physiologically acceptable carriers, diluents or excipients. Such composition may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like, carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (e.g. aluminum hydroxide) and preservatives. In addition, pharmaceutical compositions of the present invention may also contain one or more additional active ingredients, such as, for example, cytokines like B-interferon.

In this respect a further aspect of the present invention comprise pharmaceutical compositions for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising at least one immunomodulating agent and a pharmaceutically acceptable carrier, said at least one immunomodulating agent comprising at least one Fc receptor ligand and at least one immunosuppressive factor. Similarly, the invention comprises methods for the preparation of a pharmaceutical composition to treat an immune disorder comprising combining at least one immunomodulating agent with a physiologically acceptable carrier or diluent wherein said immunomodulating agent comprises at least one Fc receptor ligand and at least one immunosuppressive factor. In both of these aspects the immunosuppressive factor may comprise a T cell receptor antagonist or agonist and the Fc receptor ligand may comprise at least part of a immunoglobulin constant region domain. Preferably, the immunomodulating agent will be in the form of a recombinant polypeptide or a chimeric antibody.

As indicated above, immunomodulating agents comprising chimeric antibodies are a particularly preferred aspect of the invention. Such antibodies may be formed by substituting a immunosuppressive factor, typically a peptide TCR antagonist, for at least part of one or more of the complementarity determining regions (CDR). As will be described more fully in the Examples below, the nucleotide sequence coding for the heavy chain may be engineered to replace all or part of at least one CDR with a peptide analog of all or part of an autoantigen. Upon expression by the proper cell line, the recombinant heavy chains can complex with wild type light chains to form an

immunoreactive tetramer displaying two immunosuppressive factors. Those skilled in the art will appreciate that the immunoglobulin molecules may be selected from the species to be treated so as to minimize the generation of a harmful immune response (i.e. a human anti-mouse response). As the constant region of the selected immunoglobulin is essentially unmodified, this form of immunomodulating agent is readily endocytosed allowing for effective presentation of the associated immunosuppressive factor.

In other forms, the immunomodulating agents of the present invention may comprise an antigen-antibody complex wherein the antigen is an immunosuppressive factor. It will be appreciated that modern immunological techniques may be used to generate and purify the desired antibodies which are preferably monoclonal. By way of example only, a selected peptide antagonist or agonist (i.e. an analog of a peptide autoantigen) may be injected into a mouse to provide immunoreactive cells which may then be harvested and immortalized using standard methods. If desired, the murine monoclonal may be "humanized" using conventional recombinant procedures leaving a small murine variable region expressed on an otherwise human immunoglobulin that will not provoke a harmful immune response in a patient. In any case, the monoclonal antibody is complexed with the immunosuppressive factor to form the desired immunomodulating agent which may then be formulated and administered as described above. With the intact constant region forming the FcR ligand, phagocytation should be relatively rapid and presentation of the attached immunosuppressive factor efficient.

Although embodiments may comprise the Fc receptor ligands corresponding to the entire constant region, it must be emphasized that the present invention does not require that the administered immunomodulating agent comprise an intact immunoglobulin constant region. Rather, any FcR ligand that can bind to the FcR and undergo endocytosis may be used in conjunction with the selected immunosuppressive factor. Specifically, single domains of constant regions or fragments thereof may be combined with peptide antagonists to form monomeric polypeptides (having a single amino acid chain) that can suppress the immune system in accordance with the teachings herein. Such fusion proteins may be constructed which, having the minimum effective FcR ligand and/or immunosuppressive factor, may be much more stable thereby facilitating delivery and possibly increasing bioavailability. Moreover, these engineered proteins may be able to be administered over a period of time without provoking an immune response as is seen when administering whole antibodies of heterologous species. As such, relatively small chimeric polypeptides may prove to be effective immunomodulating agents.

Similarly, non-peptide based molecular entities may prove to be efficient FcR ligands, immunosuppressive factors or, in combination, immunomodulating agents. Those skilled in the art will appreciate that molecular entities (peptide based or non-peptide based) that function effectively in a selected role (i.e. FcR ligand) may be provided using current procedures such as combinatorial chemistry, directed evolution or rational drug design. For example, it may be possible to use rational drug design to fashion a small non-peptide molecular entity that effectively binds to a previously elucidated Fc receptor. The derived FcR ligand may then be covalently linked (or otherwise reversibly associated) with an immunosuppressive factor such as a peptide antagonist to provide an immunomodulating agent that exhibits particular stability or other desirable traits.

Whatever form of immunomodulating agent selected the compositions of the present invention may be formulated to provide desired stability and facilitate the selected form of administration. For example, the compositions may be administered using all the conventional routes including, but not limited to, oral, vaginal, aural, nasal, pulmonary, intravenous, intracranial, intraperitoneal, subcutaneous, or intramuscular administration. Within other embodiments of the invention, the compositions described herein may be administered as part of a sustained release implant. Within yet other embodiments, compositions of the present invention may be formulated as a lyophilizate or spray dried formulation, utilizing appropriate excipients which provide stability as a lyophilizate, and subsequent to rehydration.

The present invention is useful for the treatment of any vertebrate comprising an immune system subject to down regulation. The invention is particularly useful in those vertebrates such as mammals that possess cellular immune responses. In preferred embodiments the vertebrate to be treated will be in a neonatal or infant state.

In this respect, a further aspect of the invention comprises a method for treating an immune disorder comprising administering to a patient a therapeutically effective amount of a pharmaceutical composition comprising an immunomodulating agent in combination with a physiologically acceptable carrier or diluent wherein said immunomodulating agent comprises at least one Fc receptor ligand and at least one immunosuppressive factor. For this aspect, the immunosuppressive factor may comprise a T cell receptor antagonist and the Fc receptor ligand may comprise at least part of a immunoglobulin constant region domain. As previously alluded to, the immunomodulating agent will preferably be in the form of a recombinant polypeptide or a chimeric antibody. The methods may be used treat immune disorders comprising autoimmune disorders, allergic responses and transplant rejection and are particularly useful in treating autoimmune disorders selected from the group consisting of multiple sclerosis, lupis, rheumatoid arthritis, scleroderma, insulin-dependent diabetes and ulcerative colitis.

As discussed above, the compositions, compounds and methods of the present invention are particularly useful for inducing tolerance in neonatal or infant mammals thereby preventing or reducing future autoimmunity. The term "infant" as used herein, refers to a human or non-human mammal during the period of life following birth wherein the immune system has not yet fully matured. In humans, this period extends from birth to the age of about nine months while in mice, this period extends from birth to about four weeks of age. The terms "newborn" and "neonate" refer to a subset of infant mammals which have essentially just been born. Other characteristics associated with "infants" according to the present invention include an immune response which has (i) susceptibility to high zone tolerance (deletion/anergy of T cell precursors, increased tendency for apoptosis); (ii) a Th_2 biased helper response (phenotypical particularities of neonatal T cells; decreased CD40L expression on neonatal T cells); (iii) reduced magnitude of the cellular response (reduced number of functional T cells; reduced antigen-presenting cell function); and (iv) reduced magnitude and restricted type of humoral response (predominance of IgM^{high}, IgD^{low}, B cells, reduced cooperation between Th and B cells). In specific nonlimiting embodiments of the invention the disclosed immunmodulating agents may be administered to an infant mammal wherein maternal antibodies remain present in detectable amounts. In a related embodiment, the pregnant mother may be inoculated with the disclosed compositions so as to produce the desired T cell tolerance in the fetus. In any case the induced T cell tolerance

may confer resistance to the later development of an autoimmune disease associated with the administered immunomodulating agent.

Regardless as to whether the subject is an infant or full grown, the pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patients disease. Within particularly preferred embodiments of the invention, the pharmaceutical compositions described herein may be administered at a dosage ranging from 1μg to 50 mg/kg, although appropriate dosages may be determined by clinical trials. Those skilled in the art will appreciate that patients may be monitored for therapeutic effectiveness by MRI or signs of clinical exacerbation.

Following administration, it is believed that the immunomodulating agent binds to one or more Fc receptors present on the surface of at least one type of antigen presenting cell. Those skilled in the art will appreciate that selection of the FcR ligand will, at least to some extent, determine which class of Fc receptor is used to internalize the immunomodulating agent. That is, a FcR ligand corresponding to an IgG constant region will be bound by a different class of Fc receptor than a FcR ligand corresponding to an IgE constant region. Moreover, as different classes of Fc receptors are expressed on different types of antigen presenting cells it is possible to present the immunosuppressive factor on selected APCs. For example, an FcR ligand corresponding to an IgG constant region is likely to be endocytosed by a macrophage or neutrophil and presented accordingly. This is of interest in that certain APCs are more efficient at presenting various types of antigens which, in turn, may influence which T cells are activated.

In any case, the entire immunomodulating agent is subjected to receptor mediated endocytosis by the APC and usually becomes localized in clathrin-coated vesicles. After internalization, the immunomodulating agent is processed for eventual presentation at the surface of the APC. Processing generally entails vesicle transport of the immunomodulating agent to the lysosome, an organelle comprising an acidic pH and selected enzymes including proteases. Here the immunomodulating agent is digested to provide a free immunosuppressive factor which, for the purposes of the instant invention, may be in the form of a peptide. In such cases average peptide lengths may be, for example, on the order of 5 to 30 amino acids. Following digestion, at least some of the immunomodulating agent fragments, including the immunosuppressive factor fragment, are associated with MHC class II molecules in exocytic vesicles. The MHC class II-immunosuppressive factor complex is then transported to the surface of the APC and presented to helper T cells.

As pointed out above, preferred embodiments of the invention use a TCR antagonist as the immunosuppressive factor presented in concert with the class II MHC molecules. Accordingly, such antagonists (which may be peptide analogs) will be used for the purposes of the following discussion. However, it must be emphasized that the present invention may be used for the receptor mediated endocytic presentation of any immunosuppressive factor that downregulates an immune response. As such, T cell receptor agonists which provide the desired reduction in immunogenic response may be used as immunosuppressive factors and are in the purview of the present invention.

Accordingly, by way of example only, a T cell may have previously been sensitized to an autologous peptide agonist corresponding to a fragment of myelin basic protein. In multiple sclerosis this autoagonist is continuously presented thereby activating an immune response directed to constituents of the myelin sheath. More particularly, the sensitized individual T cells express thousands of receptors which selectively bind to the presented autoagonist and signal the cell. When enough of the receptors are bound, the sensitized T cell acts to mount a response i.e. secrete interleukin. In the cases where a TCR antagonist is presented in concert with MHC class II molecules the T cell will recognize the presented complex but will not be activated.

Thus, in accordance with the present invention, efficient endocytic presentation of an immunosuppressive factor (i.e. an antagonist) inhibits agonist-TCR binding through competition for the receptors. That is, the presented TCR antagonist binds effectively to the TCR of a sensitized T cell thereby precluding binding of a presented autoantigen or fragment thereof. Yet, unlike an autoantigen-TCR complex, the immunosuppressive factor-TCR complex does not signal the T cell to mount a response. Thus, the binding of the immunosuppressive factor (non-reactive agonist or antagonist) can prevent a T cell from binding enough autoantigen to reach the threshold activation level that induces the cell to act. Hence, a harmful immune response to the continuously presented autoantigen comprising a natural agonist is averted.

Presentation of the following non-limiting Examples will serve to further illustrate the principles of the present invention. In this regard, a list of abbreviations and corresponding definition used throughout the following discussion and the Examples is provided:

MBP: myelin basic protein, has been implicated in the etiology of multiple sclerosis;

PLP: proteolipid protein, has been implicated in the etiology of multiple sclerosis;

PLP1: a peptide fragment of PLP comprising aa residues 139-151;

PLP-LR: a peptide analog of PLP1, does not activate PLP1 pulsed cells;

PLP2: a peptide fragment of PLP comprising aa residues 178-191;

Ig-W: an Ig construct (used herein as a control) comprising the heavy chain variable region of the anti-arsonate antibody 91A3, linked to a Balb/cγ2b constant region, and the parental 91A3 kappa light chain;

Ig-PLP1: the same construct as Ig-W except that the heavy chain CDR3 was replaced with aa residues 139-151 of PLP;

Ig-PLP-LR: the same construct as Ig-W except that the heavy chain CDR3 was replaced with a peptide analog of aa residues 139-151 of PLP;

Ig-HA: (used as a control herein) the same construct as Ig-W except that the heavy chain CDR3 was replaced with aa residues 110-120 of influenza virus HA;

PPD: purified protein derivative, whole *Mycobacterium tuberculosis* extract used as a control activator.

For obvious practical and moral reasons, initial work in humans to determine the efficacy of experimental compositions or methods with regard to many diseases is infeasible. Thus, during early development of any drug it is standard procedure to employ appropriate animal models for reasons of safety and expense. The success of implementing laboratory animal models is predicated on the understanding that immunodominant epitopes are

frequently active in different host species. Thus, an immunogenic determinant in one species, for example a rodent or pig, will generally be immunoreactive in a different species such as in humans. Only after the appropriate animal models are sufficiently developed will clinical trials in humans be carried out to further demonstrate the safety and efficacy of a vaccine in man. Accordingly, for purposes of explanation only and not for purposes of limitation, the present invention will be primarily demonstrated in the exemplary context of mice as the mammalian host. Those skilled in the art will appreciate that the present invention may be practiced with other mammalian hosts including humans and domesticated animals.

In this respect, experimental encephalomyelitis (EAE), which is used as an animal model for MS, can be induced in susceptible strains of mice with myelin autoantigens such as PLP and myelin basic protein (MBP). The encephalitogenic activity of these proteins correlates with the presence of peptides which induce in vivo class II restricted encephalitogenic T cells and consequently EAE. The peptide corresponding to aa residues 139-151 of PLP (PLP1) is encephalitogenic in H-2s SJL mice, and T cell lines specific for PLP1 transfer EAE into naive animals. Although the target antigen(s) in human MS is still debatable, the frequency of T cells specific for myelin proteins are higher in MS patients than in normal subjects. Silencing those myelin-reactive T cells may be a logical approach to reverse MS. As such, this model will be used to demonstrate the advantages of the present invention.

Example I

Preparation of Peptides

For the purposes of this application the amino acids are referred to by their standard three-letter or one-letter code. Unless otherwise specified, the L-form of the amino acid is intended. When the 1-letter code is used, a capital letter denotes the L-form and a small letter denotes the D-form. The one letter code is as follows: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

All peptides used in the following examples were produced by Research Genetic, Inc. (Huntsville, Alabama) using solid state methodology and purified on HPLC columns to >90% purity using conventional methods. PLP1 peptide (HSLGKWLGHPNKF: SEQ. ID No. 1) encompasses an encephalitogenic sequence corresponding to aa residues 139-151 of naturally occurring proteolipid protein. PLP-LR (HSLGKLLGRPNKF:SEQ. ID No. 2) is an analog of PLP1 in which Trp144 and His147 were replaced with Leu and Arg (underlined), respectively. PLP1 and PLP-LR bind well to I-A^S class II molecules (i.e. an MHC class II structure produced by a specific strain of mice). PLP2 peptide (NTWTTCQSIAFPSK:SEQ. ID No. 3) encompasses an encephalitogenic sequence corresponding to aa residues 178-191 of PLP. This peptide also binds to I-A^S class II molecules and induces EAE in SJL mice. HA peptide (sequence not shown) corresponds to aa residues 110-120 of the hemagglutinin of the Influenza virus. HA binds to I-E^D class II molecules and is used here as control peptide.

Example II

Production of Murine Chimeric Immunoglobulins Comprising Exogenous Peptides

Two immunoglobulin-peptide chimeras, designated Ig-PLP1 and Ig-PLP-LR and shown schematically in Figure 1, were constructed to express peptides PLP1 and PLP-LR as described in Example 1. In both cases, the heavy chain CDR 3 loop was deleted and replaced with nucleotide sequences coding for the selected peptide. Conventional DNA sequencing analysis indicated insertion of peptide nucleotide sequences in the correct reading frame.

The genes used to construct these chimeras include the gene coding for the BALBK IgG_2b constant region as described by Gillian et al., *Cell.* 33:717,1983, the gene coding for the 91A3 heavy chain variable region as described by Ruthban et al., *J. Mol. Bio.*, 202:383-398, 1988, and the gene coding for the entire 91A3 kappa light chain as described by Gary et al., *Proc. Natl. Acad. Sci.*, 84:1085-1089, 1987, all of which are incorporated herein by reference. The procedures for deletion of the heavy chain CDR3 region and replacement with nucleotide sequences coding for PLP1 and PLP-LR are similar to those described by Zaghouani et al. *J.Immunol.* 148: 3604-3609, 1992 and incorporated herein by reference, for the generation of Ig-NP a chimera carrying a CTL epitope corresponding to aa residues 147-161 of the nucleoprotein of PR8 influenza A virus. The same reference reports that the CDR3 of the 91A3 IgG is compatible for peptide expression, and that both class I and class II-restricted epitopes have been efficiently processed and presented to T cells when grafted in place of the naturally occurring segment.

Briefly, The $91A3V_H$ gene was subcloned into the EcoRI site of pUC19 plasmid and used as template DNA in PCR mutagenesis reactions to generate $91A3V_H$ fragments carrying PLP1 ($91A3V_H$-PLP1) and PLP-LR ($91A3V_H$-PLP-LR) sequences in place of CDR3. Nucleotide sequencing analysis indicated that full PLP1 and PLP-LR sequences were inserted in the correct reading frame (not shown). The $91A3V_H$-PLP1 and $91A3V_H$-PLP-LR fragments were then subcloned into the EcoRI site of pSV2-gpt-Cγ2b in front of the exons coding for the constant region of a Balb/cγ2b which generated pSV2-gpt-$91A3V_H$-PLP1-Cγ2b and pSV2-gpt-$91A3V_H$-PLP1-LR-Cγ2b plasmids, respectively. These plasmids were then separately cotransfected into the non-Ig producing SP2/0 B myeloma cells with an expression vector carrying the parental 91A3 light chain, pSV2-neo-91A3L. Transfectants producing Ig chimeras were selected in the presence of geneticin and mycophenolic acid. Transfectants were cloned by limiting dilution and final clones secreted 1 to 4 μg/mL of Ig-PLP1 or Ig-PLP-LR (collectively, the Ig-PLP chimeras). The selected cell lines, designated Ig-PLP1-9B11 and Ig-PLP-LR-21A10, are maintained in permanent storage in the inventor's laboratory.

Chimeric and wild-type antibodies were also used as controls. For example Ig-HA, an IgG molecule carrying in place of the D segment the HA110-120 T helper epitope from the HA of influenza virus that differs from Ig-PLP1 and Ig-PLP-LR only by the peptide inserted within CDR3. Ig-W is the product of unmodified (wild-type) $91A3V_H$ gene, Balb/cγ2b constant region and 91A3 kappa light chain. Therefore it differs from Ig-PLP1 and Ig-PLP-LR in the CDR3 region which comprises the parental D segment. Finally, Ig-PLP2, is a chimeric antibody that carries within the heavy chain CDR3 loop aa residues 178-191 of PLP. Conventional cloning, sequencing, and purification procedures were used to generate the appropriate cell lines and are similar to those described by Zaghouani et al. (previously cited)

and those previously used to generate Ig-HA, Zaghouani et al., *Science*. 259:224-227, 1993 also incorporated herein by reference.

Large scale cultures of transfectants were carried out in DMEM media containing 10% iron enriched calf serum (Intergen, New York). Ig-PLP chimeras were purified from culture supernatant on columns made of rat-anti-mouse kappa chain mAb and coupled to CNBr activated Sepharose 4B (Pharmacia). Rat-anti-mouse kappa chain mAb (RAM 187.1 or ATCC denotation, HB-58) and mouse anti-rat kappa light chain mAb (MAR 18.5 or ATCC denotation, TIB 216) were obtained from the ATCC. These hybridomas were grown to large scale and purified from culture supernatant on each other. The rat anti-mouse kappa mAb was used to prepare the columns on which the Ig-PLP chimeras were purified from culture supernatant. To avoid cross contamination separate columns were used to purify the individual chimeras.

Example III

Purification of Proteolipid Protein

Native proteolipid protein or PLP was purified from rat brain according to the previously described procedure of Lees et al., in Preparation of Proteolipids, *Research Methods in Neurochemistry*, N. Marks and R. Rodnight, editors. Plunemum Press, New York, 1978 which is incorporated herein by reference.

Briefly, brain tissue was homogenized in 2/1 v/v chloroform/methanol, and the soluble crude lipid extract was separated by filtration through a scintered glass funnel. PLP was then precipitated with acetone and the pellet was redissolved in a mixture of chloroform/methanol/acetic acid and passed through an LH-20-100 sephadex column (Sigma) to remove residual lipids. Removal of chloroform from the elutes and conversion of PLP into its apoprotein form were carried out simultaneously through gradual addition of water under a gentle stream of nitrogen. Subsequently, extensive dialysis against water was performed to remove residual acetic acid and methanol.

Example IV

Production of Rabbit Anti-Peptide Antibodies

PLP1 and PLP-LR peptides prepared in Example I were coupled to KLH and BSA as described in Zaghouani et al., *Proc. Natl. Acad. Sci USA*. 88:5645-5649, 1991 and incorporated herein by reference. New Zealand white rabbits were purchased from Myrtle's Rabbitry (Thompson Station, TN). The rabbits were immunized with 1 mg peptide-KLH conjugates in complete Freund's adjuvant (CFA) and challenged monthly with 1 mg conjugate in incomplete Freund's adjuvant (IFA) until a high antibody titer was reached. The peptide-BSA conjugates were coupled to sepharose and used to purify anti-peptide antibodies from the rabbit anti-serum.

Example V

Characterization of Rabbit Anti-Peptide Antibodies

Capture radioimmnoassays (RIA) were used to assess expression of PLP1 and PLP-LR peptides on an IgG molecule using Ig-PLP1 and Ig-PLP-LR made as described in Example II.

Microtiter 96-well plates were coated with the rabbit anti-peptide antibodies made in Example IV (5 μg/mL) overnight at 4°C and blocked with 2% BSA in PBS for 1 hour at room temperature. The plates were then washed 3 times with PBS, and graded amounts of Ig-PLP1 and Ig-PLP-LR were added and incubated for 2 hours at room temperature. After 3 washes with PBS, the captured Ig-PLP1 and Ig-PLP-LR were detected by incubating the plates with 100 x 10^3 cpm ^{125}I-labeled rat anti-mouse kappa mAb for 2 hours at 37°C. The plates were then washed 5 times with PBS and counted using an LKB gamma counter. Shown are the mean ± SD of triplicates obtained with 27 μg/mL of chimeras.

As shown in Figure 2, the rabbit antibodies directed to synthetic PLP1 and PLP-LR peptides recognized the chimeric antibodies Ig-PLP1 and Ig-PLP-LR produced in Example II. More specifically, when Ig-PLP1 and Ig-PLP-LR were incubated on plates coated with rabbit anti-PLP1 they were captured in significant quantity and bound labeled rat anti-mouse kappa chain mAb (Fig. 2A). Similarly, both Ig-PLP1 and Ig-PLP-LR were captured by rabbit anti-PLP-LR (Fig. 2B). Conversely, Ig-W, the wild type 91A3 murine antibody without an exogenous peptide and an IgM control antibodies (not shown), did not show significant binding to the rabbit antibodies. Ig-PLP1 bound to both anti-PLP1 and anti-PLP-LR better than did Ig-PLP-LR, indicating that structural differences affected accessibility of the peptides to the rabbit antibodies. Further, the results shown in Figure 2 indicate that peptide expression on the chimeras did not alter heavy and light chain pairing because the rabbit antibodies bind to the PLP peptide on the heavy chain and the labeled rat anti-mouse kappa binds on the light chain.

Example VI

Antigen Specific T Cell Line Proliferation Assays

PLP1-specific T cell hybridomas 5B6 and 4E3 and the IL-2 dependent HT-2 T helper cells were obtained from The Eunice Kennedy Shriver Center, Waltham, MA. The 5B6 and 4E3 T cells recognize the peptide PLP1 in association with I-A^S class II MHC and produces IL-2 when incubated with it as reported by Kuchroo et al., *J. Immunol.* 153:3326-3336, 1994 which is incorporated herein by reference. Conversely, Kuchroo et al. report that when stimulated with PLP1 and then with PLP-LR both 5B6 and 4E3 cells no longer produce IL-2. Similarly, stimulation of T cell hybridomas with PLP1 in the presence of PLP-LR apparently inhibits IL-2 production.

Using substantially the same technique as Kuchroo et al., activation of the T cell hybridomas for various agonists was performed as follows. Irradiated (3,000 rads) splenocytes from SJL mice were used as antigen presenting cells (APCs) for this Example. The irradiated splenocytes were incubated in 96-well round bottom plates (5 x 10^5 cells/well/50 μl) with graded concentrations of antigens (100 μl/well). After one hour, T cell hybridomas, i.e. 5B6 or 4E3 (5 X 10^4 cells/well/50 μl) were added and the culture was continued overnight. Activation (or proliferation) of the T cells was assessed by measuring production of IL-2 in the culture supernatant. This was done

by ^{3}H-thymidine incorporation using the IL-2 dependent HT-2 cells. That is, when IL-2 is present (i.e. secreted by activated T cells) the HT-2 cells proliferate, incorporating labeled thymidine from the surrounding media.

The culture media used to carry out these assays was DMEM supplemented with 10% FBS, 0.05 mM 2-mercaptoethenol, 2 mM glutamine, 1 mM sodium puryvate and 50 μg/mL gentamycin sulfate. Briefly, culture supernatants (100 μl/well) were incubated with HT-2 cells (1x 10^4 cells/well/100 μl) in 96-well flat bottom plates for 24 hours. Subsequently 1 μCi ^{3}H-thymidine was added per well and the culture was continued for an additional 12-14 hours. The cells were then harvested on glass fiber filters and the non incorporated ^{3}H-thymidine was washed away. Incorporated thymidine was then counted using the trace 96 program and an Inotech β counter. It will be appreciated that those wells containing higher levels of IL-2 (secreted by the activated T cell hybridoma lines) will induce higher levels of HT-2 cell proliferation and register increased levels of ^{3}H-thymidine incorporation.

The results of the aforementioned assay using two different T cell lines are shown in Figure 3. Specifically, T cell hybridomas 4E3 (Fig. 3A) and 5B6 (Fig. 3B) produced substantial levels of IL-2 following stimulation by APCs previously incubated with Ig-PLP1, PLP1 and native PLP. The negative controls Ig-W, Ig-HA, and PLP2 peptide did not induce the production of IL-2 by the T cells. Similarly, both Ig-PLP-LR and PLP-LR peptide did not stimulate 5B6 and 4E3 to produce significant levels of IL-2. These last results are not unexpected because the PLP-LR peptide is known to negate rather than stimulate IL-2 production. The concentration of antigen was 0.1 μM for Ig-PLP1, Ig-PLP-LR, Ig-HA, and Ig-W; 1 μM for PLP1, and PLP2 peptides; and 1.7 μM for PLP. Each value represents the mean $\pm$ SD of triplicate wells.

These results indicate that Ig-PLP1 was presented to the T cell hybridomas in a manner conducive to activation. Steric hindrance appears to preclude the simultaneous direct binding of the whole antibody to the MHC structure and TCR. As T cells will not react to soluble proteins, it appears that the PLP1 peptide was released from the Ig by endocytic processing and bound MHC class II I-A^s molecules. Accordingly, the regions flanking the PLP1 peptide do not appear to interfere with the endocytic processing of Ig-PLP1 or the binding of the PLP1 peptide to the MHC class II structure.

Example VII

Presentation of PLP1 Peptide to T Cells Via Ig-PLP1

In spontaneous immune disorders, exposure and continuous endocytic presentation of an autoantigen may generate significant levels of MHC-autoantigen complexes. Currently many immune diseases lack an effective *in vitro* model for replicating this continuous presentation affording a serious impediment to the development of effective treatments. Due to relatively inefficient internalization mechanisms or the previously discussed limitations relating to free peptides, relatively high levels of natural antigens are required to provide the desired stimulation. Accordingly, one aspect of the present invention is to provide an *in vitro* model for the continuous endocytic presentation of agonist ligands.

More particularly, the present invention provides methods for the effective *in vitro* endocytic presentation of a T cell antagonist comprising the steps of:

a. providing a medium comprising a plurality of antigen presenting cells expressing Fc receptors; and

b. combining said medium with a immunomodulating agent containing composition wherein the composition comprises an immunomodulating agent having at least one Fc receptor ligand and at least one immunosuppressive factor and a compatible carrier.

Preferably the immunosuppressive factor will be at least one T cell receptor antagonist and the Fc receptor ligand will be at least part of a immunoglobulin constant region domain. Further, in preferred aspects of the invention the immunomodulating agent will comprise a recombinant polypeptide or a chimeric antibody.

In this respect, Ig-PLP1 (or any immunoglobulin associated agonist) may be used for the purpose of establishing a peptide delivery system that could efficiently operate through the endocytic pathway and generate high levels of agonist ligands such that it provides an *in vitro* system to investigate the immune system. In particular, the disclosed system may be used to investigate antagonism in a situation similar to the *in vivo* presentation of autoantigens.

To demonstrate that immunoglobulin associated agonists may be used to mimic continuous endocytic presentation of antigens, T cell activation assays were performed with free PLP1 peptide, native PLP, and Ig-PLP1. The results of the assays are shown in Fig. 4.

Specifically, different concentrations of the three antigens (i.e. agonists) were incubated with irradiated SJL/J splenocytes which were subsequently associated with 4E3 T cell hybridomas. IL-2 production was measured by ^{3}H-thymidine incorporation using the IL-2 dependent HT-2 cells as described in Example VI. Each point represents the mean of triplicates. The standard deviation did not exceed 10% of the mean value.

Fig. 4 shows that, although the maximum activation levels varied among the three different agonists, the levels required to stimulate the T cells were much lower for Ig-PLP1 than for either free PLP1 or native PLP. That is, it took substantially less Ig-PLP1 to stimulate the cell line than either the native PLP or the free peptide (on the order of 1/100). Specifically, stimulation to half the maximum level required less Ig-PLP1 (0.005 μM) than PLP (0.5 μM) or PLP1 peptide (0.6 μM). These results indicate that the PLP1 T cell epitope is better presented by Ig-PLP1 than by native PLP or by synthetic PLP1 peptide. Although the plateau of IL-2 production was higher when the T cell activator is free PLP1 synthetic peptide it requires substantially higher agonist levels that may be difficult to obtain *in vivo* over an extended period.

While not limiting the present invention in any way, it appears that the efficacy of Ig-PLP1 in peptide delivery is related to FcR mediated internalization and access to newly synthesized MHC molecules. More particularly, native PLP appears to internalize rather ineffectively by simple fluid phase pinocytosis while free PLP1 peptide appears to simply bind to empty MHC class II molecules at the cell surface. The ineffectual presentation of these forms of the autoantigen is clearly illustrated by Fig. 4 which unambiguously shows that Ig-PLP1 is more efficient in presenting PLP1 peptide in combination with MHC class II molecules than either the free peptide or the native protein.

Example VIII

Inhibition of T Cell Activation *In vitro*

Antagonism of PLP1, PLP, and Ig-PLP1 T cell activation by Ig-PLP-LR was detected using a prepulsed proliferation assay.

Irradiated (3,000 rads) SJL splenocytes (used as APCs) were incubated in 96-well round bottom plates (5 x 10^5 cells/well/50 μl) with the selected agonist (1 μM PLP1 peptide, 0.05 μM Ig-PLP1 or 7 μM PLP) and various concentrations of antagonist (100 μl/well) for 1 hour. Subsequently, 4E3 T cell hybridomas (5 X 10^4 cells/well/50 μl) were added and the culture was continued overnight. IL-2 production in the supernatant, determined as in Example VI using HT-2 cells, was used as measure of T cell activation. The results of this assay are shown in Figure 5.

More particularly, Figures 5A, 5B and 5C show antagonism of free PLP1 peptide (5A), Ig-PLP1 chimeric immunoglobulin (5B) and native PLP (5C) respectively. The antagonists were Ig-PLP-LR (squares) and PLP-LR (circles) with controls of Ig-W (diamonds) and PLP2 (triangles).

Cpm values obtained when the APCs were incubated with the agonist but no antagonist was used as control thymidine incorporation. This value was 7,503 ± 1,302 for Ig-PLP1; 31,089 ± 3,860 for PLP1 peptide; and 8,268 ± 915 for PLP. The cpm value obtained when the APCs were incubated with no agonist or antagonist was used as background (BG). This value was 1,560 ± 323 for Ig-PLP1; 2,574 ± 290 for PLP1 peptide; and 2,127 ± 177 for PLP. The percent control thymidine incorporation was calculated as follows: [(cpm obtained in the presence of test antagonist) - (BG)] / [(cpm control thymidine incorporation value) - (BG)]. Each point represents the mean of triplicates.

As previously discussed, the potency of Ig-PLP1 chimeras in peptide loading onto MHC class II molecules may resemble *in vivo* autoimmune circumstances where a continuous supply of antigen often allows for abundant generation of self peptides which can trigger T cell aggressively. Figure 5A (PLP1 agonist) shows that when T cells were incubated with APCs in the presence of both PLP1 and Ig-PLP-LR, a substantial decrease in IL-2 production occurred as the concentration of Ig-PLP-LR increased. A similar decline in IL-2 production was evident when the synthetic PLP-LR peptide was used during T cell activation with PLP1 peptide. Conversely, antagonistic effects were not observed with the control Ig-W immunoglobulin and the PLP2 peptide. Inhibition of IL-2 production to half the maximum level (60% control thymidine incorporation) required only 0.4 μM Ig-PLP-LR versus 9 μM PLP-LR peptide indicating a much more efficient presentation of, and T cell antagonism by, Ig-PLP-LR.

Further evidence that the chimeric immunoglobulin is more efficient than the free peptide in T cell antagonism is shown in Figs. 5B and 5C. Specifically, Fig. 5B shows that Ig-PLP-LR inhibited T cell activation mediated by Ig-PLP1 while free PLP-LR, like the negative control PLP2 peptide, did not show any significant antagonism. Significantly, Fig. 5B also shows that Ig-W, the wild type 91A3 immunoglobulin without any exogenous peptide exhibits partial inhibitory activity in Ig-PLP1 mediated T cell activation. It is believed that this may be the result of competition for binding to the FcR on the APCs because both Ig-PLP1 and Ig-W share identical IgG2b constant regions. A maximum of 50% inhibition in IL-2 production was seen when the activation of T cells by Ig-

PLP1 was carried out in the presence of Ig-W. Thus, Ig-W would compete with Ig-PLP1 for FcR binding and internalization thereby diminishing the activation of T cells. That is, as the concentration of Ig-W increases, less Ig-PLP1 will bind to FcR and be internalize by the APCs resulting in a diminished presentation and corresponding IL-2 production. It is important to note that this Ig-W mediated reduction in response is not the result of antagonistic effects but rather simply a result of competition for FcR binding. That is, the presented Ig-W epitopes are not TCR antagonists for PLP1 and do not interact with the PLP1 specific TCRs.

In contrast to Fig. 5B, Fig. 5C shows that Ig-PLP-LR, but not Ig-W, significantly reduces the activation of T cells by native PLP. As Ig-W is likely internalized in a different manner than native PLP, (Fc receptor versus simple fluid phase pinocytosis) there should not be any direct competition for uptake and processing and hence no inhibition.

For the sake of convenience the results shown in Figure 5 are summarized in Table 1 immediately below. When APCs were incubated with PLP1 peptide in the presence of Ig-PLP-LR there was no activation of the PLP1-specific T cell hybridomas (Figure 5a). Moreover, when the activation of T cells by native PLP and Ig-PLP1 was carried out in the presence of various concentrations of Ig-PLP-LR, IL-2 production (i.e. T-cell activation) declined as Ig-PLP-LR increased. However, free PLP-LR peptide failed to inhibit T cell activation mediated by native PLP or Ig-PLP1. These two lines of evidence indicate that the principal mechanism for Ig-PLP-LR mediated inactivation of T cells was likely to be endocytic presentation and TCR antagonism rather than direct blockage of MHC class II molecules on the cell surface.

In the table below a plus sign indicates inhibition of IL-2 production and therefore antagonism, while a minus sign indicates little or no inhibition of IL-2 production and therefore little or no antagonism.

Table 1.

Ig-PLP-LR and PLP-LR Mediated T Cell Antagonism.

	Stimulator (Agonist)		
Antagonist	**PLP1**	**PLP**	**Ig-PLP1**
PLP-LR	+	-	-
Ig-PLP-LR	+	+	+

The results of the foregoing example indicate that the FcR mediated uptake and subsequent processing of a peptide antagonist are compatible with efficient presentation by the antigen presenting cell. This is extremely

unexpected in view of the prior art where the delivery of free peptide analogs was assumed to provide efficient antagonism through direct competition for MHC or TCR binding sites.

Example IX

Characterization of Mechanism for Antagonism by Ig-PLP-LR

Using an assay similar to the one performed in Example VIII, it was demonstrated that competition for direct binding to the Fc receptor is not, in and of itself, a likely mechanism for Ig-PLP-LR mediated antagonism.

SJL splenic APCs were incubated with native PLP (6.8 μM) in the presence of 2 μM Ig-PLP2, Ig-PLP-LR, or Ig-W and assayed for IL-2 production by ^{3}H-thymidine incorporation using HT-2 cells as described in the previous Examples. Ig-PLP2 was prepared as in Example II using the sequence detailed in Example I. The % control thymidine incorporation was calculated as in Example VIII. Results of the assay are shown in Fig. 6 wherein each column represents the mean ± SD of triplicates.

As with the results shown in Fig. 5B, the present Example supports the position that both efficient presentation on the MHC class II structure and an effective peptide analog provide the most significant results. That is, even though the Ig-PLP2 chimeric antibody is taken up and processed, efficient presentation of the PLP2 peptide by I-A^S will not preclude activation of the T-cells as it is not an analog of the native PLP agonist. Accordingly, simple competition binding to MHC class II molecules on the antigen presenting cells is not likely to produce the desire antagonism.

Example X

In vivo Induction of a T Cell Response to PLP1

By this Example it was demonstrated that, in addition to generating a T cell response *in vitro* (Example VII), the chimeric antibodies of the present invention could be used to generate a cellular response *in vivo*. Specifically, the following Example demonstrates the *in vivo* priming of PLP1 specific T cells by Ig-PLP1.

Six to eight week old SJL mice (H-2^S) were purchased from Harlan Sprague Dawley (Frederick, MD) and maintained in an animal facility for the duration of experiments.

The mice were immunized subcutaneously in the foot pads and at the base of the limbs and tail with 50 μg of Ig-PLP1 emulsified in a 200 μl mixture of 1:1 v/v PBS/CFA. Ten days later the mice were sacrificed by cervical dislocation, the spleens and lymph nodes (axillary, inguinal, popliteal, and sacral) were removed, single cell suspension were prepared, and the T cell responses were analyzed. The results shown in Figure 7 are those obtained with 4 x 10^5 lymph node cells/well (7A) and 10 x 10^5 spleen cells/well (7B). The activators PLP1 and PLP2 were used at 15 μg/mL and PPD was used at 5 μg/mL.

As with the previous Examples, T cell activation was monitored using a proliferation assay comprising ^{3}H-thymidine incorporation. Here, lymph node and spleen cells were incubated for three days in 96-well round bottom plates, along with 100 μl of a single selected activator, at 4 and 10 x 10^5 cells/100 μl/well, respectively. Subsequently, 1 μCi ^{3}H-thymidine was added per well, and the culture was continued for an additional 12-14 hours.

The cells were then harvested on glass fiber filters, and incorporated ^{3}H-thymidine was counted using the trace 96 program and an Inotech β counter. A control media with no stimulator was included for each mouse and used as background.

Each value shown in Figure 7 was calculated as described in Example VIII and represents the mean ± SD of triplicates after deduction of background cpms obtained with no activator in the media. Similar results were obtained when mice were immunized with 150 μg of Ig-PLP per mouse (not shown).

Figures 7A and 7B clearly show that, when Ig-PLP1 was injected subcutaneously in the foot pads and at the base of the limbs and tail, a strong specific T cell response to the PLP1 peptide was induced. While there was some variation as to the strength of the reaction among the individual mice, the lymph node and spleen cells of each produced a significant response upon challenge with the PLP1 peptide. Interestingly there is a significant PLP1 specific response detected in the spleen, an organ that mostly filters and responds to systemic antigens. One possibility that can be put forth to explain these results is that Ig-PLP1, because of it's long half life, was able to circulate and reach both the lymphatic and blood circulation and consequently be presented at both systemic and lymphatic sites. This is potentially very beneficial when implementing therapeutic regimens for autoimmune disorders. It was also interesting that some mice show proliferation when the cells are stimulated with PLP2 peptide *in vitro*. Possibly, the fact that this peptide is presented by I-A^S like PLP1 allows low affinity cells to bind and generate a response. In any case the results are consistent with those provided by the earlier Examples where it was shown that Ig-PLP1 was efficient in presenting the peptide to T cells *in vitro*.

Example XI

In vivo Inhibition of a T Cell Response to PLP1

As seen in the previous Example, Ig-PLP1 is capable of priming T cells *in vivo* and generates a potent immune response when exposed to the agonist PLP1 peptide. This Example demonstrates that the administration of a peptide antagonist in the form of a chimeric antibody immunomodulating agent can substantially reduce the immune response generated by the endocytic presentation of an agonist ligand. Specifically, this Example demonstrates that co-administration of Ig-PLP-LR with Ig-PLP1 significantly reduces the immune response to PLP1 peptide.

Mice were co-immunized with mixtures of either 50 μg Ig-PLP1 and 150 μg Ig-PLP-LR or 50 μg Ig-PLP1 combined with 150 μg Ig-W. In particular, individual mice from three groups (4 mice per group) were injected sc. as in Example X with a 200 μl mixture (PBS/CFA, 1:1 v/v) containing one of the following mixtures: 50 μg Ig-PLP1 and 150 μg Ig-PLP-LR; 50 μg Ig-PLP1 and 150 μg Ig-W; or Ig-PLP1 and 100 μg PLP-LR peptide. Splenic and lymph node T cell responses were analyzed at day 10 post immunization using the protocol set forth in Example X. The lymph node cells were assayed at 4 x 10^5 cells/well and the spleen cells at 10 x 10^5 cells/well. The agonist ligand was PLP1 at 15 μg/mL. Results for the lymph node and spleen cells, shown in Figs. 8A and 8B respectively and summarized in Table 2 below, represent the mean ± SD of triplicates after deduction of background cpm obtained with no agonist in the media.

Figures 8A and 8B show that, although Ig-PLP1 was efficiently presented and induced a strong *in vivo* T cell response (Example X), it was possible to antagonize such a response by including Ig-PLP-LR in the mixture administered to mice. Indeed, when Ig-PLP1 was co-administered to mice with Ig-PLP-LR, the subsequent immune response to free PLP1 peptide was markedly reduced as shown on the right half of Figs. 8A and 8B. It appears that the low PLP1 response for both the spleen and lymph node tissue was a result of PLP-LR antagonism, since the co-administration with Ig-PLP1 of the wild type antibody, Ig-W, did not significantly reduce the T cell response. These results strongly indicate that it is the efficient *in vivo* presentation of PLP-LR through the FcR binding and endocytic processing of Ig-PLP-LR that is responsible for the reduced cellular response.

Moreover, as seen in Table 2 immediately below, when free PLP-LR peptide was co-administered with Ig-PLP1 there was no indication that the PLP1 response was reduced. The numbers provided in the table represent the percentage values of PLP1 specific proliferation relative to PPD specific proliferation and were derived as follows: (mean cpm of triplicates obtained with PLP1 stimulation - mean cpm triplicate BG) /(mean cpm of triplicates obtained with PPD - mean cpm triplicate BG) x 100

Table 2

Ig-PLP-LR But Not Free PLP-LR Peptide Mediates T Cell Antagonism In Vitro

	Ig-PLP1 co-administered with:		
Mouse	**Ig-W**	**Ig-PLP-LR**	**PLP-LR peptide**
		PLP1/PPD (%)	
1	**100**	**28**	**81**
2	**95**	**40**	**91**
3	**78**	**37**	**93**
4	**79**	**25**	**100**

The results above clearly show that co-administration of the free antagonist peptide or the control Ig-W lacking an antagonist peptide have little effect on the generated immune response. The lack of antagonist effect by free PLP-LR peptide was not due to a net lower amount of injected peptide because the mice were given approximately 34 fold more PLP-LR in the free peptide form than in the Ig-PLPLR form (on the basis of a MW of 150,000 D, the 150 μg of Ig-PLP-LR given to the mice correspond to 1 nmole of Ig that contains 2 nmoles of PLP-LR peptide, while with a MW of 1,468 Daltons the 100 μg of free PLP-LR peptide corresponds to 68 nmoles of

peptide). The failure of PLP-LR peptide to inhibit Ig-PLP1 mediated T cell activation coupled with the potency of Ig-PLP-LR in antagonizing Ig-PLP1 T cell stimulation supports the belief that Ig-PLP-LR mediated *in vivo* antagonism is likely related to efficient presentation.

Example XII

Induction of a T Cell Response to an Endocytically Presented Antagonist

Previous Examples have shown that administration of chimeric antibodies comprising a agonist ligand can prime immune cells *in vivo*. It was also shown that administration of a chimeric antibody comprising an antagonist can reduce a subsequent response to challenge by an agonist ligand. This Example demonstrates that efficient presentation of an antagonist can prime immune cells *in vivo* and mount a strong response that could effect the reaction of the T cells to an agonist peptide. Specifically, mice co-injected with Ig-PLP1 and Ig-PLP-LR develop a relatively high proliferative response to PLP-LR and practically no response to PLP1 peptide.

Lymph node and spleen cells were obtained in the same manner as set forth in Example X following co-administration of Ig-PLP1 and Ig-PLP-LR. Proliferative responses in individual mice were also measured using the methods set out in the previous Example following in vitro stimulation with either free PLP1 peptide or PLP-LR peptide at 15 μg/mL. The results of the assays using lymph node and spleen cells are detailed in Figures 9A and 9B respectively.

As can be seen from Figure 9, both spleen and lymph nodes developed responses to the antagonist PLP-LR but not to the PLP agonist PLP1. Knowing that Ig-PLP-LR induced PLP-LR specific T cells when it was co-administered with Ig-PLP1, it can be speculated that these PLP-LR-specific T cells downregulate PLP1 specific T cells. Conversely, although there was induction of PLP-LR-specific response when free PLP-LR peptide was administered with Ig-PLP1 (not shown), there was no evident reduction in the proliferative response to PLP1. Accordingly, the data set forth in the instant example demonstrates that the use of chimeric antibodies comprising an antagonist are much more effective for modulating the immune response to an antigen agonist than the free peptide antagonist.

More particularly, in view of the foregoing examples it appears that TCR engagement with PLP-LR-I-A^S complexes (i.e. MHC-PLP-LR complexes) on the surface of APCs antagonizes T cells rather than stimulates them. Accordingly, antagonism by Ig-PLP-LR may occur because efficient presentation of Ig-PLP-LR in endocytic vacuoles ensures significant levels of PLP-LR-I-A^S complexes (antagonist complexes) are generated. The amount of complexes on the cell surface is proportional to the amount of Ig-PLP-LR offered to the APCs. When PLP1 stimulation is carried out in the presence of Ig-PLP-LR, both PLP-LR-I-A^S and PLP1-I-A^S are present on the surface of a given APC where an increase in the concentration of Ig-PLP-LR leads to higher number of PLP-LR-I-A^S complexes. It will be appreciated that approximately 3500 TCR have to be engaged in order for a T cell to be activated and that a given complex of MHC class II-peptide complex serially engages approximately 200 TCRs. As such, it appears that a T cell is antagonized when TCR engagement with PLP-LR-I-A^S complexes override engagement with the agonist PLP1-I-A^S. Overall, because of efficient loading of PLP-LR by Ig-PLP-LR, T cell antagonism is achieved by a higher frequency of serial triggering of TCR by PLP-LR-I-A^S complexes. That is, the efficient uptake and processing of Ig-PLP-LR simply

means that too many of the surface MHC complexes present the PLP-LR antagonist to allow the remaining surface complexes presenting the PLP1 agonist ligand to engage the number of TCRs to activate the T cell. Therefore, the T cells will not be activated as long as the antagonist is presented at a rate that ensures the activation concentration of MHC class II-agonist complexes is not reached on the APC.

Example XIII

Lymph Node Proliferative Responses to Immunization With Ig-PLP Chimeras

Proliferative responses were measured in mice immunized with individual Ig-PLP chimeras or varying mixtures of Ig-PLP1 and Ig-PLP-LR. It was observed that Ig-PLP-LR given alone to mice induced T cells which, like those induced by Ig-PLP1, cross-reacted with both PLP1 and PLP-LR peptides. Surprisingly, however, despite the cross-reactivity of the responses, when the chimeras were administered together they displayed a dose dependent antagonism on one another resulting in down-regulation of both T cell responses. Finally, antigen specific T cells induced either by IG-PLP 1 or by IG-PLP-LR were refractory to down-regulation by peptide mixtures and proliferated significantly when they were in vitro stimulated simultaneously with both PLP1 and PLP-LR. These findings indicate that both agonist and antagonist peptides exert adverse reactions on one another and reveal an anti-parallel antagonism and a stringent control of TCR triggering at the level of naive T cells.

Materials were obtained and mice immunized as described above. Proliferative responses were measured by thymadine incorporation as set forth in Example VI above. Lymph node and spleen cells were obtained in the same manner as set forth in Example X following co-administration of Ig-PLP1 and Ig-PLP-LR. Mice were injected with 50 μg Ig-PLPI (10A), 50 μg Ig-PLP-LR (10B), 100 μg PLP1 (10C) or 100 μg PLP-LR (10D) in CFA, and 10 days later the lymph node cells were in vitro stimulated with the indicated free peptides. The stimulators PLP1, PLP-LR and PLP2 were used at the defined optimal concentration of 15 μg/ml.

The data illustrated in figs 10A-10D indicate that Ig-PLP1, like PLP1 peptide, induced a specific T cell response to PLP1 peptide. Similarly, Ig-PLP-LR, like PLP-LR peptide, induced a specific T cell response to PLP-LR peptide. Neither the Ig chimera nor the free peptides induced T cells that significantly reacted with the negative control PLP2, a peptide that is also presented by I-A^s class II molecules. Surprisingly, however, the response induced by Ig-PLP1 cross-reacted with PLP-LR peptide, while the response induced by Ig-PLP-LR cross-reacted with PLP1. The responses induced with free PLP1 or free PLP-LR were not cross-reactive.

Example XIV

Lymph Node T cell Proliferative Response to
Co-Immunization With Ig-PLP1 and Ig-PLP-LR

Mice were injected with the indicated chimeras and 10 days later the lymph nodes cells were in vitro stimulated with free peptides, and assayed for proliferation by [^{3}H]thymidine incorporation as detailed above. The results are shown in Fig. 11.

The number preceding the Ig chimera label indicates the μg amount injected per mouse. The stimulators were PPD, 5 μg/ml; PLP 1, PLP-LR, and PLP2 at 15 μg/ml. Cells incubated without stimulator were used as background (BG). The mice were tested individually and triplicate wells were assayed for each stimulator. To standardize the results and eliminate intrinsic individual variability we expressed the results as relative proliferation estimated as follows: (mean test peptide cpm - mean BG cpm)/(mean PPD cpm - mean BG cpm). The indicated relative proliferation represents the mean ± SD of 5 mice tested individually. The mean cpms ± SD obtained with PPD stimulation for the different groups of mice were as follows: 50μg Ig-PLP1:16,413 ± 1330; 50μg Ig-PLP-LR: 11,224 ± 3481; 50μg Ig-W: 11,513 ± 1,572; 50μg Ig-PLP1 + 50μg Ig-PLP-LR: 16,817 ± 2,869; 50μg Ig-PLP1 + 150μg Ig-PLP-LR: 16,156 ± 2006; 50μg Ig-PLP1 + 150μg Ig-W: 11,699 ± 1,142; 50μg Ig-PLP-LR + 150μg Ig-W: 13,435 ± 1,650; 50μg Ig-PLP1 + 50μg Ig-PLP2: 10,056 ± 1,407; and 50μg Ig-PLP-LR + 50μg Ig-PLP2: 10,877 ± 563. Filled and hatched bars indicate proliferation to PLP1 and PLP-LR respectively. The proliferation to PLP2 peptide was at background levels except where Ig-PLP2 was used in the immunization mixture.

As can be seen in Figure 11, lymph node T cells from a group of mice that were immunized with Ig-PLP1 proliferated equally well to PLP1 and to PLP-LR whereas Ig-W control caused little reaction. Surprisingly, the PLP-LR response was at background levels. Accordingly, although the responses to the Ig chimeras share cross-reactivity between PLPL and PLP-LR peptides, the mixture yielded down regulation rather than additive responses. In fact, the data suggest an anti-parallel down regulation among Ig-PLP1 (agonist) and Ig-PLP-LR (antagonist). This down-regulation appeared to be dose dependent because mice that were injected with a mixture of 50 μg Ig-PLP1 and 150 μg Ig-PLP-LR failed to respond to PLPI and mounted responses to PLP-LR that were reduced to levels observed with mice injected with Ig-PLP1 alone.

One possible explanation for the observed opposite down regulation between IG-PLPI and Ig-PLP-LR is that clonal expansion requires an optimal serial triggering with an homogeneous peptide (i.e. all or most of the receptors on a single naive T cell must engage one type of peptide in order to expand). Simultaneous stimulation of naive t cells with peptides encompassing subtle differences at the TCR contact residues, which may be occurring during immunizations involving mixtures of Ig-PLP1 and Ig-PLP-LR, fails to cause T cell expansion and in vitro proliferation.

Example XV

Splenic Proliferative T Cell Responses of Mice
Co-Immunized with Ig-PLP1 and IG-PLP-LR

As shown in Figure 12, spleen cells from the mice described in Example XIV were stimulated with PLP1 (filled bars) and PLP-LR (hatched bars.) in triplicate wells and proliferation was measured as above. The results were standardized as above using PPD cpms obtained with lymph node T cells because the proliferation of spleen cells upon stimulation with PPD was minimal. The indicated relative proliferation represents the meant ± SD of 5 individually tested mice.

Splenic T cells from these mice failed to respond to PLP-LR stimulation. However, when an additional group of mice was immunized with Ig-PLP-LR, both lymph node and splenic cells proliferated to PLP1 as well as to PLP-LR

peptide. In the spleen, although the proliferative responses were much lower than in the lymph nodes, additive responses were still not observed. Rather, an opposite down-regulatory effect between Ig-PLP1 and Ig-PLP-LR was observed. Although co-injection of Ig-W with either Ig-PLP1 or Ig-PLP-LR did not affect either response, co-injection of Ig-PLP2 with Ig-PLP1 increased reactivity to PLP-LR among the T cells induced by Ig-PLP1.

Example XVI

IL-2 Production by Splenic Cells of Mice

Co-Immunized With Ig-PLP1 and Ig-PLP-LR

To further investigate the opposing down regulation among Ig-PLP1 and Ig-PLP-LR, splenic antigen induced cytokine responses were measured in animals immunized with either a single or both Ig-chimeras. As shown in Fig. 13, spleen cells (1 X 10^6 per well) from the mice described in Example XIV were stimulated with PLP1 (filled bars) and PLP-LR (hatched bars) for 24 hours. Production of IL-2 (13A), INFγ (13B), and IL-4 (13C) were measured as set forth below.

Cells were incubated in 96 well round-bottom plates at 10 x 10^5 cells/100μl/well with 100μl of stimulator, as above, for 24 hours. Cytokine production was measured by ELISA according to Pharmingen's instructions using 100μl culture supenatant. Capture antibodies were rat anti-mouse IL-2, JES6-IAI2;rat anti-mouse IL-4, 11B11;rat anti-mouse IFNγ, R4-6A2; and rat anti-mouse IL10, JES5-2A5. Biotinylated anti-cytokine antibodies were rat anti-mouse IL-2, JES6-5H4; rat ant imouse IL-4, BVD6-24G2; rat anti-mouse IFNγ, XMG 12; and rat anti-mouse IL- 10, JES5-16E3) The OD405 was measured on a Spec 340 counter (Molecular Devices) using SOH MAX PRO version 1.2.0 software. Graded amounts of recombinant mouse IL-2, IL-4, INFγ, and IL-10 were included in all experiments in order to construct standard curves. The concentration of cytokines in culture supernatants was estimated by extrapolation from the linear portion of the standard curve. Cells incubated without stimulator were used as background (BG). Each mouse was individually tested in triplicate wells for each stimulator and the indicated cpms represent the mean ± SD after deduction of BG cpms. Production of IL-10 was also measured, but the results were at background levels (not shown).

Upon in vitro stimulation with PLP1 peptide, T cells from Ig-PLP1 immunized mice produced IL-2, INFγ, and small amounts of IL-4. However, stimulation of the same cells with PLP-LR yielded minimal IL-2 and undetectable INFγ or IL-4. Spleen cells from Ig-PLP-LR immunized mice generated IL-2 but no IFNγ or IL-4 upon stimulation with PLP1 peptide. Moreover, PLP-LR peptide stimulation produced only a minimal IL-2 response. In mice immunized with equal amounts of Ig-PLP1 and Ig-PLP-LR all cytokine production was reduced to minimal or background levels upon stimulation with either peptide. Co-immunization of Ig-W with either chimera had no measurable effect on cytokine production pattern. When the animals were given a 3:1 ratio of Ig-PLP-LR: Ig-PLP1, although the splenic proliferative responses and IL-2 production were at background levels, significant amounts of IL-4 and INFγ were evident upon stimulation with PLP-LR peptide. Consequently, the excess of Ig-PLP-LR may lead to a mixed but PLP-LR dominant TCR triggering that induces cells able to produce cytokine but which exhibit no proliferative response. These data

indicated that Ig-PLP1 and Ig-PLP-LR exerted adverse reactions on one another leading to down-regulation of both T cell responses.

Example XVII

Proliferation of Antigen Experienced T Cells Upon Stimulation In Vitro With Mixtures of PLPI and PLP-LR Peptides

To investigate whether Ig-PLP1 and Ig-PLP-LR could display adverse reactions on each other at the level of antigen experienced cross-reactive T cells, mice were immunized with Ig-PLP1 or Ig-PLP-LR alone and assessed for proliferative T cell responses upon *in vitro* stimulation with varying mixtures of free PLPI and PLP-LR peptides.

More particularly Mice (4 per group) were immunized with 50μg Ig-PLP1 (14A and 14B) or 50μg Ig-PLP-LR (14C and 14D) in CFA, and 10 days later the lymph node (14A and 14C) and spleen (14B and 14D) cells were stimulated with the indicated peptides and assayed for [^{3}H]thymidine incorporation as above. The number preceding the peptide label indicates the μg/ml amount used for in vitro stimulation. The specific proliferation was estimated by deducting the mean BG (obtained by incubating cells without stimulator) cpm from the test sample cpm. The indicated cpms represent the mean ± SD of 4 individually tested mice. ND, not determined.

As can be seen in Figs. 14A-14D, both lymph node and spleen cells from mice immunized with Ig-PLPI or Ig-PLP-LR proliferated equally as well to stimulation with a single peptide as to a mixture of PLPL and PLP-LR. The proliferative response to the mixture,in most cases, was even higher than the response to a single peptide stimulation.

Example XVIII

IL-2 Production by Antigen Experienced T Cells Upon In Vitro Stimulation With PLP1/PLP-LR Peptide Mixtures

To further investigate whether Ig-PLP1 and Ig-PLP-LR could display adverse reactions on each other at the level of antigen experienced cross-reactive T cells, mice were immunized with Ig-PLP1 or Ig-PLP-LR alone and assessed for cytokine responses upon *in vitro* stimulation with varying mixtures of free PLPI and PLP-LR peptides. The results are shown in Figs. 15A and 15B.

Spleen cells from Ig-PLP1 (15A) and Ig-PLP-LR (15B) immunized mice were stimulated with the indicated peptides and tested for IL-2 production by ELISA as in Example XVI. The spleen cells used in these experiments were from the mice described in Example XVII. The number preceding the name of the peptide represents the μg/mi amount used for stimulation. The indicated μg/ml IL-2 values represent the mean d: SD of 4 individually tested mice.

As indicated by Example XVII, IL-2 production was not decreased upon stimulation of spleen cells with varying mixtures of PLP1 and PLP-LR. To the contrary, in most cases of stimulation with peptide mixture IL-2 production was higher than in stimulation with a single peptide. Again these findings indicate that both agonist and antagonist peptides exert adverse reactions on one another and reveal an anti-parallel antagonism and a stringent control of TCR triggering at the level of naive T cells.

In addition to the use of immunomodulating agents comprising T cell receptor antagonists and agonists for attenuation of adult immune responses, the same compositions may advantageously be used for the induction of tolerance in neonates and infants as demonstrated in the following Examples.

5

Example XIX

ISJL/J Mice Injected with Ig-PLP1 at Birth
Resist Induction of EAE During Adult Life

To demonstrate the advantages of inoculating neonates or infants with the compositions of the present invention, newborn mice were administered immunomodulating agents as described herein and exposed to agents for the inducement of an autoimmune condition.

More specifically, neonatal mice (10 mice per group) were injected with 100 μg of affinity chromatography purified Ig-PLP1 or Ig-W within 24 hours of birth and were induced for EAE with free PLP1 peptide at 7 weeks of age. Mice were scored daily for clinical signs as follows: 0, no clinical signs; 1, loss of tail tone; 2 , hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or death. Panel A shows the mean clinical score of all mice and panel B shows the mean score of the surviving animals only. EAE was induced by subcutaneous injection in the foot pads and at the base of the limbs and tail with a 200 μl IFA/PBS (1vol/1vol) solution containing 100 μg free PLP1 peptide and 200 μg *M. tuberculosis* H37Ra. Six hours later 5 x 10^9 inactivated B. *pertussis* were given intravenously. After 48 hours another 5 x 10^9 inactivated *B. pertussis* were given to the mice.

As may be seen in Figs 16A and 16B adult mice recipient of Ig-PLP1 in saline at birth resisted the induction of EAE by free PLP1 peptide. Indeed, the clinical scores were much less severe in those mice than in animals recipient of Ig-W, the parental wild type Ig without any PLP peptide. In addition, contrary to those mice which received Ig-W, mice injected with Ig-PLP1 showed no relapses (figure 16B).

Example XX

In Vivo Presentation of Ig-PLP1 by Neonatal
Thymic and Splenic Antigen Presenting Cells

In order to confirm the clinical results observed in Example XX, cytokine responses were measured in neonatal mice. The data obtained is shown in Fig. 17.

Specifically, neonates (5 mice per group) were injected with 100 μg Ig-PLP1 or Ig-W within 24 hours of birth. Two days later the mice were sacrificed, and pooled thymic (17A) and splenic (17B) cells were irradiated and used as APCs for stimulation of the PLP1-specific T cell hybridoma 4E3 as described above. IL-2 production in the supernatant which was used as a measure of T cell activation was determined using the IL-2 dependent HT-2 cell line as described by V.K. Kuchroo et al. *J. Immunol.* 153, 3326 (1994) incorporated herein by reference. The indicated cpms represent the mean ± SD of triplicates.

The administered Ig-PLP1 was efficiently presented by neonatal APCs. Both thymic (17A) and splenic (17B) APCs from neonate recipients of IG-PLP1 activated a T cell hybridoma specific for PLP1 peptide without addition of erogenous antigen. APCs from neonate recipients of Ig-W were unable to activate the T cell hybridoma.

Example XXI

Reduced Splenic Proliferative T cell

Response in Mice Recipient of Ig-PLP1 at Birth

To further confirm the results observed in the previous two Examples, proliferative responses were measured in mice inoculated with an immunomodulating agent at birth. The results are shown in Figs. 18A and 18B.

Neonates were injected intraperitoneal (i.p.)within 24 hours of birth with 100 μg Ig-PLPI or Ig-W in saline. When the mice reached 7 weeks of age they were immunized with 100 μg free PLP1 peptide in 200 μl CFA/PBS (1vol/1vol) s.c. in the foot pads and at the base of the limbs and tail. Ten days later the mice were sacrificed, and (18A) the lymph node (0.4 x 10^6 cells/well) and (18B) the splenic (1 X 10^6 cells/well) cells were in vitro stimulated for four days with 15 μg/ml free PLP1 or PLP2, a negative control peptide corresponding the encephalitogenic sequence 178-191 of PLP (13). One μCi/well of[^{3}H]thymidine was added during the last 14.5 hours of stimulation, and proliferation was measured using an Inotech β-counter and the trace 96 Inotech program. The indicated cpms represent the mean ± SD of triplicate wells for individually tested mice. The mean cpm ± SD of lymph node proliferative response of all mice recipient of Ig-PLP1 and Ig-W was 34,812 ± 7,508 and 37,026 ± 10,133, respectively. The mean splenic proliferative response was 3,300 ± 3,400 for the Ig-PLP1 recipient group and 14,892 ± 4,769 for the Ig-W recipient group.

Mice recipient of Ig-PLP1 at the day of birth, like those injected with Ig-W, developed equivalent adult lymph node T cell proliferative responses to PLP1 when they were immunized with free PLPI peptide in CFA (18A). However, the splenic proliferative response was markedly reduced in the mice recipient of Ig-PLP1 (18B) thus indicating the inducement of tolerance. Neither group of mice showed a significant proliferative response to PLP2, a negative control peptide presented by I-A^s class II molecules like PLP1.

Example XXII

Lymph Node T Cell Deviation in Mice Treated With Ig-PLP1 at Birth

To further demonstrate the induction of tolerance in infants or neonates, cytokine responses were measured in were measured in mice inoculated with an immunomodulating agent at birth. The results are shown in Figs. 19A-19C.

In particular, lymph node cells (4 x 10^5 cells/well) from the mice described in Example XXI were stimulated *in vitro* with free PLP1 or PLP2 (15 μg/ml) for 24 hours, and the production of IL-2 (19A), IL-4 (19B), and INFγ (19C) was measured by ELISPOT as described in Example XVI using Pharmingen anti-cytokine antibody pairs. The indicated values (spot forming units) represent the mean ± SD of 8 individually tested mice.

The results show cytokine production patterns were affected by the inoculation of the neonatal mice. Lymph node cells from mice recipient of Ig-W at birth produced, upon stimulation with PLP1, IL-2 but not INFγ or IL-4. In contrast, cells from mice recipient of Ig-PLP1 were deviated and instead produced IL-4. No cytokine production was observed upon stimulation with PLP2 peptide.

Example XXIII

Reduced INFγ Production by Splenic T Cells From
Mice Injected With Ig-PLP1 at the Day of Birth

To confirm the results obtained in Example XXII, spleen cells from the same mice were assayed for cytokine responses. The results are shown in Figs. 20A and 20B.

More specifically, splenic cells (1 X 10^6 cells/well) from the mice were stimulated *in vitro* with free PLP1 or PLP2 (15 μg/mi) for 24 hours, and the production of IL-2 (20A), IL-4 (20B), and INFγ (20C) in the supernatant was measured by ELISA using pairs of anti-cytokine antibodies from Pharmingen according to the manufacture's instructions (Example XVI). The indicated amounts of cytokine represent the mean ± SD of 8 individually tested mice.

In the spleen, while cells from mice innnoculate with Ig-W produced IL-2 and INFγ. Conversely, cells from mice injected with Ig-PLP1 produced IL-2 but failed to produce detectable levels of INFγ. The negative control, PLP2 peptide, failed to induce cytokine production.

Example XXIV

Cytokine Mediated Restoration of Splenic T Cell
Proliferation in Mice Injected With Ig-PLP1 at Birth

To demonstrate that proliferative responses may be retstored, cells from inoculated neonatal mice were exposed to exogonous INFγ. The results are shown in Fig. 21.

In particular, a group of neonates injected i.p. with 100 μg of Ig-PLP1 at birth were immunized with 100 μg PLP1 peptide in CFA, as in Example XXI, and *in vitro* stimulation of splenic cells (1 x 10^6 cells/well) with free PLP1 peptide (15 μg/ml) was carried out as described in Example XXI but in the presence of 100 units INFγ or IL-12. The indicated cpms for each mouse represent the mean ± SD of triplicate wells.

Surprisingly, addition of erogenous INFγ to splenic cells from the mice recipient of Ig-PLP1 at birth restored the proliferative response. IL-12, an inducer of INFγ (14), also restored the splenic proliferative response.

Overall, mice injected at birth with Ig-PLP1 develop a lymph node T cell deviation and an unusual INFγ-mediated splenic anergy. Interestingly, when these mice were induced for EAE with free PLP1 peptide they developed a mild monophasic disease without relapses. Since Igs have long half-lives, an Ig based immunomodulating agent may endure for an extended period of time resulting in a continuous and slow release of the immunosuppressive factor, as may occur in the usual neonatal tolerization procedures using incomplete Freund's adjuvant with a conventional antigen. Consequently, delivery on Igs may allow one to circumvent the use of adjuvant to induce neonatal tolerance. Further, internalization of an immunosuppressive factor via FcR and the subsequent processing in the endocytic pathway grants access to newly synthesized MHC class II molecules, generating significant amounts of MHC-immunosuppressive factor complexes. These favorable parameters (i.e. FcR-mediated APCs activation, slow peptide release, and efficient peptide presentation), may contribute to the induction of lymph node deviation and

splenic anergy. As with administration of the disclosed compositions to adults, the adjuvant free tolerization strategy may be used to silence autoreactive T cells and prevent autoimmunity.

Those skilled in the art will further appreciate that the present invention may be embodied in other specific forms without departing from the spirit or central attributes thereof. In that the foregoing description of the present invention discloses only exemplary embodiments thereof, it is to be understood that other variations are contemplated as being within the scope of the present invention. Accordingly, the present invention is not limited to the particular embodiments which have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the invention.

WHAT IS CLAIMED IS:

1. An immunomodulating agent for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising at least one Fc receptor ligand and at least one immunosuppressive factor.

2. The immunomodulating agent of claim 1 wherein said immunosuppressive factor is selected from the group consisting of T cell receptor antagonists, T cell receptor agonists and combinations thereof.

3. The immunomodulating agent of claim 2 wherein said immunosuppressive factor comprises a peptide antagonist.

4. The immunomodulating agent of claim 3 wherein said peptide antagonist is an analog of a peptide agonist capable of activating a T cell response to proteolipid protein.

5. The immunomodulating agent of claim 1 wherein said at least one Fc receptor ligand comprises at least part of a domain of a constant region of an immunoglobulin molecule.

6. The immunomodulating agent of claim 1 wherein the immunomodulating agent comprises a polypeptide.

7. The immunomodulating agent of claim 1 wherein the immunomodulating agent comprises an antibody-antigen complex.

8. The immunomodulating agent of claim 1 wherein the immunomodulating agent is a chimeric antibody.

9. The immunomodulating agent of claim 8 wherein said chimeric antibody comprises a T cell receptor antagonist.

10. The immunomodulating agent of claim 9 wherein said T cell receptor antagonist is expressed within at least one complementarity determining region.

11. A pharmaceutical composition for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising a compound as set forth in any one of claims 1-10.

12. Use of an immunomodulating agent as set forth in any one of claims 1-10 for the preparation of a pharmaceutical composition to treat an immune disorder in a patient in need thereof.

13. The method of claim 12 wherein said immune disorder comprises a disorder selected from the group consisting of autoimmune disorders, allergic responses and transplant rejection.

14. The method of claim 13 wherein said immune disorder comprises an autoimmune disorder selected from the group consisting of multiple sclerosis, lupis, rheumatoid arthritis, scleroderma, insulin-dependent diabetes and ulcerative colitis.

15. The method of claim 12 wherein said patient is an infant or neonate.

16. Use of an immunomodulating agent as set forth in any one of claims 1-10 for the preparation of a pharmaceutical composition for the induction of T cell tolerance in a patient in need thereof.

17. The method of claim 16 wherein said T cell tolerance is associated with an autoimmune disorder selected form the group consisting of multiple sclerosis, lupus, rheumatoid arthritis, sclerdoerma, insulin-dependent diabetes and ulcerative colitis.

18. The method of claim 16 wherein said patient is an infant or neonate.

19. A method for treating an immune disorder comprising:

administering to a patient a therapeutically effective amount of a pharmaceutical composition comprising an immunomodulating agent in combination with a physiologically acceptable carrier or diluent wherein said immunomodulating agent comprises at least one Fc receptor ligand and at least one immunosuppressive factor.

20. The method of claim 19 wherein said immunosuppressive factor is selected from the group consisting of a T cell receptor antagonist, a T cell receptor agonist and combinations thereof.

21. The method of claim 19 wherein said immunosuppressive factor is an analog of a peptide agonist capable of activating a T cell response to proteolipid protein.

22. The method of claim 19 wherein said immunosuppressive factor is an analog of a peptide agonist capable of activating a T cell response to myelin basic protein.

23. The method of claim 19 wherein said Fc receptor ligand comprises at least part of one domain of a constant region of an immunoglobulin molecule.

24. The method of claim 23 wherein the immunoglobulin molecule is human IgG molecule.

25. The method of claim 19 wherein said immunomodulating agent comprises a polypeptide.

26. The method of claim 25 wherein said immunomodulating agent comprises a chimeric antibody.

27. The method of claim 19 wherein said immune disorder comprises a disorder selected from the group consisting of autoimmune disorders, allergic responses and transplant rejection.

28. The method of claim 27 wherein said immune disorder comprises an autoimmune disorder selected from the group consisting of multiple sclerosis, lupis, rheumatoid arthritis, scleroderma, insulin-dependent diabetes and ulcerative colitis.

29. A method for producing an immunomodulating agent for the endocytic presentation of an immunosuppressive factor on the surface of an antigen presenting cell of a vertebrate comprising the steps of:

transforming or transfecting suitable host cells with a recombinant polynucleotide molecule comprising a nucleotide sequence which encodes a polypeptide comprising at least one Fc receptor ligand and at least one immunosuppressive factor;

culturing the transformed or transfected host cells under conditions in which said cells express the recombinant polynucleotide molecule to produce said polypeptide wherein the polypeptide comprises at least a part of an immunomodulating agent; and

recovering said immunomodulating agent.

30. The method of claim 29 wherein said immunosuppressive factor is selected from the group consisting of a T cell receptor antagonist, a T cell receptor agonist and combinations thereof.

31. The method of claim 29 wherein said immunosuppressive factor is an analog of a peptide agonist capable of activating a T cell response to myelin basic protein.

32. The method of claim 29 wherein said Fc receptor ligand comprises at least a part of one domain of a constant region of an immunoglobulin molecule.

33. The method of claim 29 wherein said immunomodulating agent comprises a chimeric antibody.

34. The method of claim 33 wherein said chimeric antibody comprises a heavy chain wherein at least one complementarity determining region has been replaced with a T cell receptor antagonist.

35. A recombinant polynucleotide molecule encoding a polypeptide wherein said polynucleotide molecule comprises at least one nucleotide sequence corresponding to a Fc receptor ligand and at least one nucleotide sequence corresponding to an immunosuppressive factor.

36. The polynucleotide molecule of claim 35 wherein said immunosuppressive factor is selected from the group consisting of a T cell receptor antagonist, a T cell receptor agonist and combinations thereof.

37. The polynucleotide molecule of claim 35 wherein said polynucleotide molecule comprises a sequence corresponding to at least part of one domain of a constant region of an immunoglobulin molecule.

38. The polynucleotide molecule claim 37 wherein the immunoglobulin molecule is a human IgG molecule.

39. The polynucleotide molecule of claim 35 wherein said polynucleotide molecule encodes a nucleotide sequence corresponding to an immunoglobulin heavy chain wherein a complementarity determining region has been at least partially deleted and replaced with a nucleotide sequence corresponding to T cell receptor antagonist.

40. Transfected or transformed cells comprising a recombinant polynucleotide molecule according to any one of claims 35 to 39.

41. A method for the effective *in vitro* endocytic presentation of an immunosuppressive factor comprising the steps of:

providing a medium comprising a plurality of antigen presenting cells expressing Fc receptors; and

combining said medium with a immunomodulating agent containing composition wherein the composition comprises an immunomodulating agent having at least one Fc receptor ligand and at least one immunosuppressive factor and a compatible carrier.

42. The method of claim 41 wherein said Fc receptor ligand comprises at least part of one domain of a constant region of an immunoglobulin molecule.

43. The method of claim 41 wherein said immunomodulating agent comprises a polypeptide.

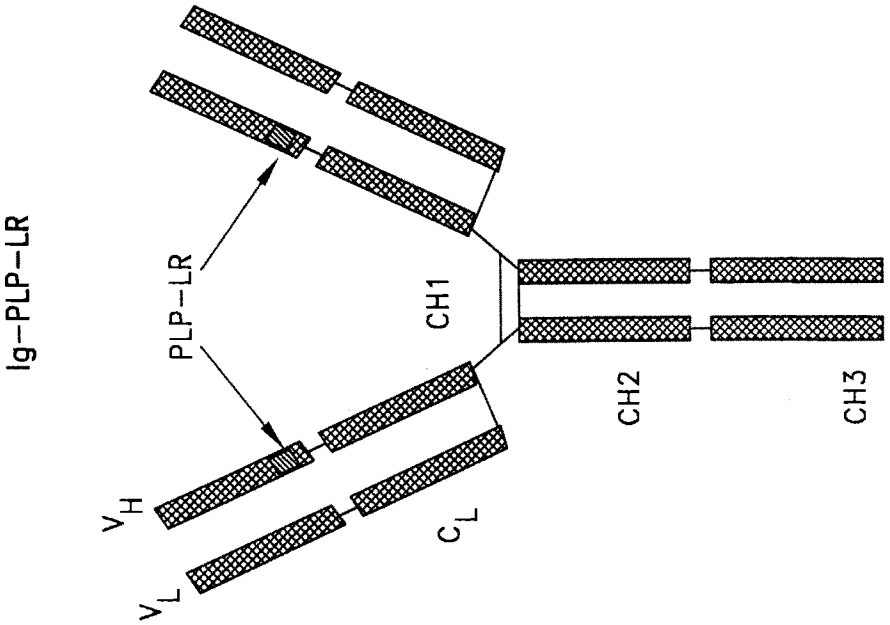

Ig-PLP-LR
PLP-LR
CH1
CH2
CH3
V_H
V_L
C_L

FIG. 1B

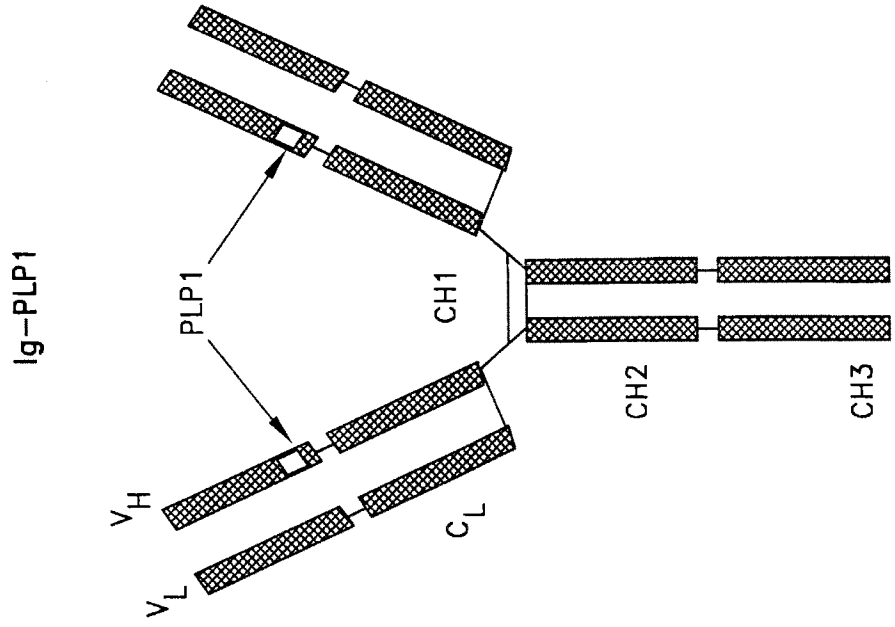

Ig-PLP1
PLP1
CH1
CH2
CH3
V_H
V_L
C_L

FIG. 1A

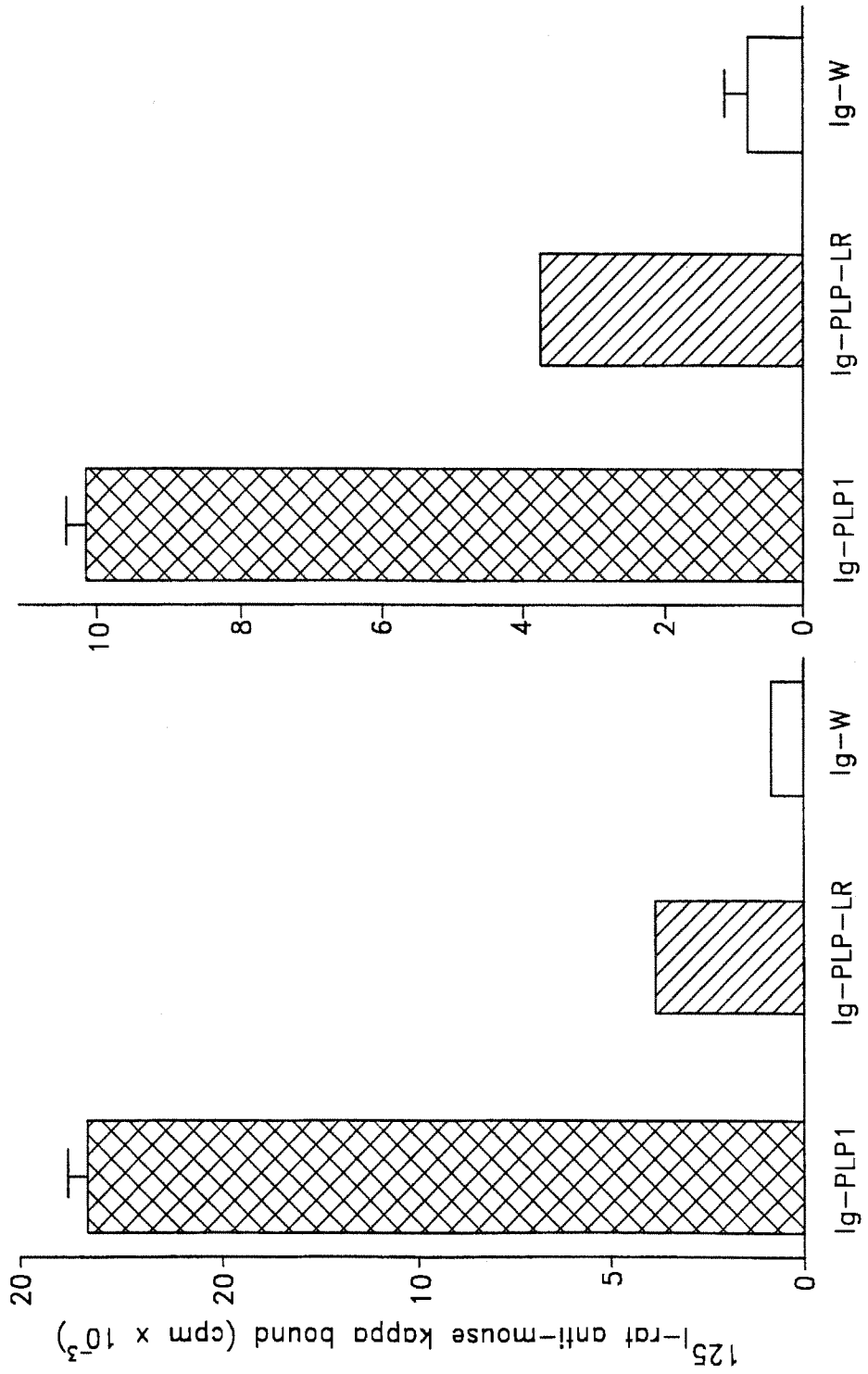

^{125}I-rat anti-mouse kappa bound (cpm x 10^{-3})
20
20
10
5
0
Ig-PLP1
Ig-PLP-LR
Ig-W
10
8
6
4
2
0
Ig-PLP1
Ig-PLP-LR
Ig-W

FIG.2A

FIG.2B

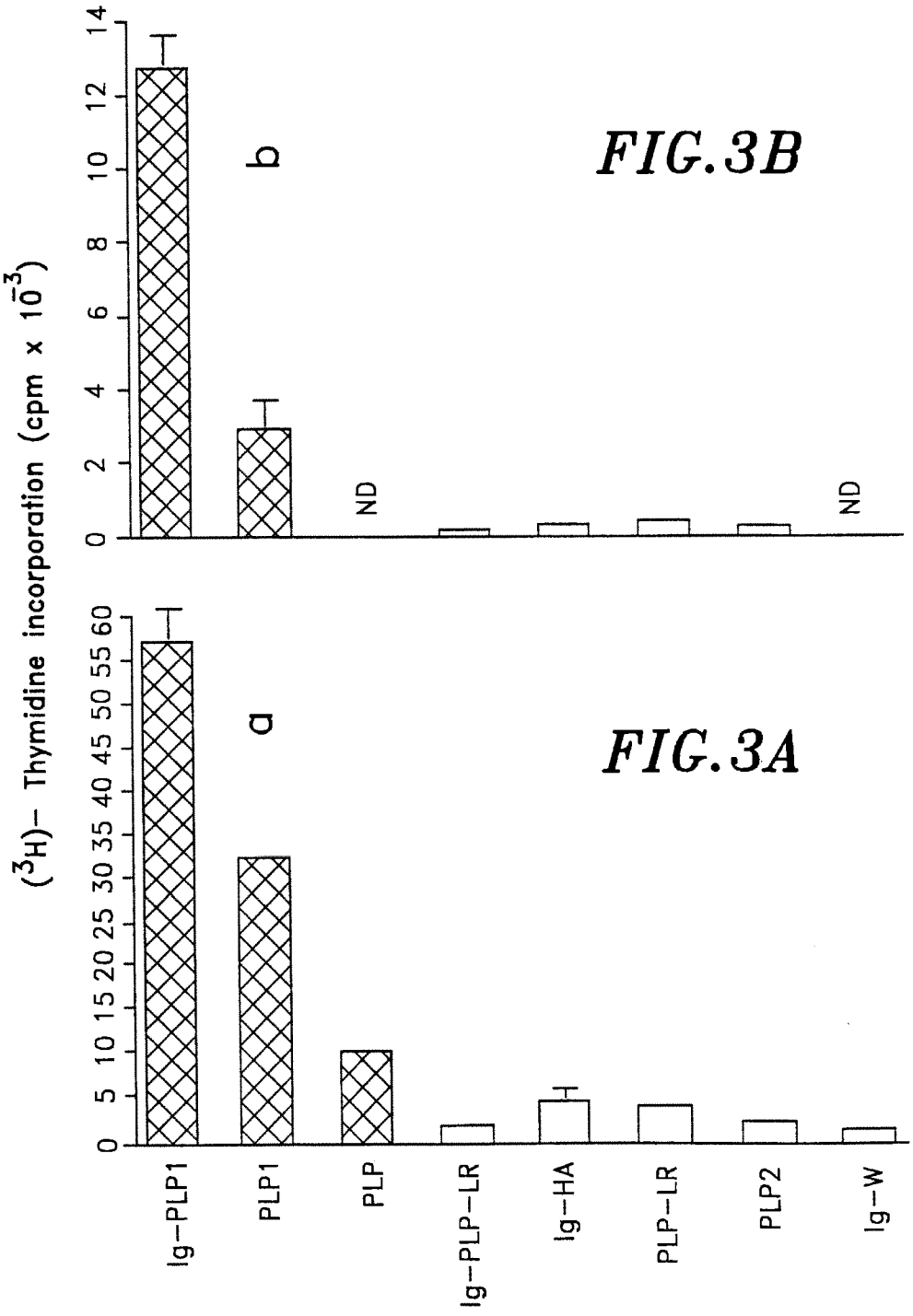

(^{3}H)- Thymidine incorporation (cpm x 10^{-3})
0 2 4 6 8 10 12 14
b
ND
ND
0 5 10 15 20 25 30 35 40 45 50 55 60
a
Ig-PLP1
PLP1
PLP
Ig-PLP-LR
Ig-HA
PLP-LR
PLP2
Ig-W

FIG.3B

FIG.3A

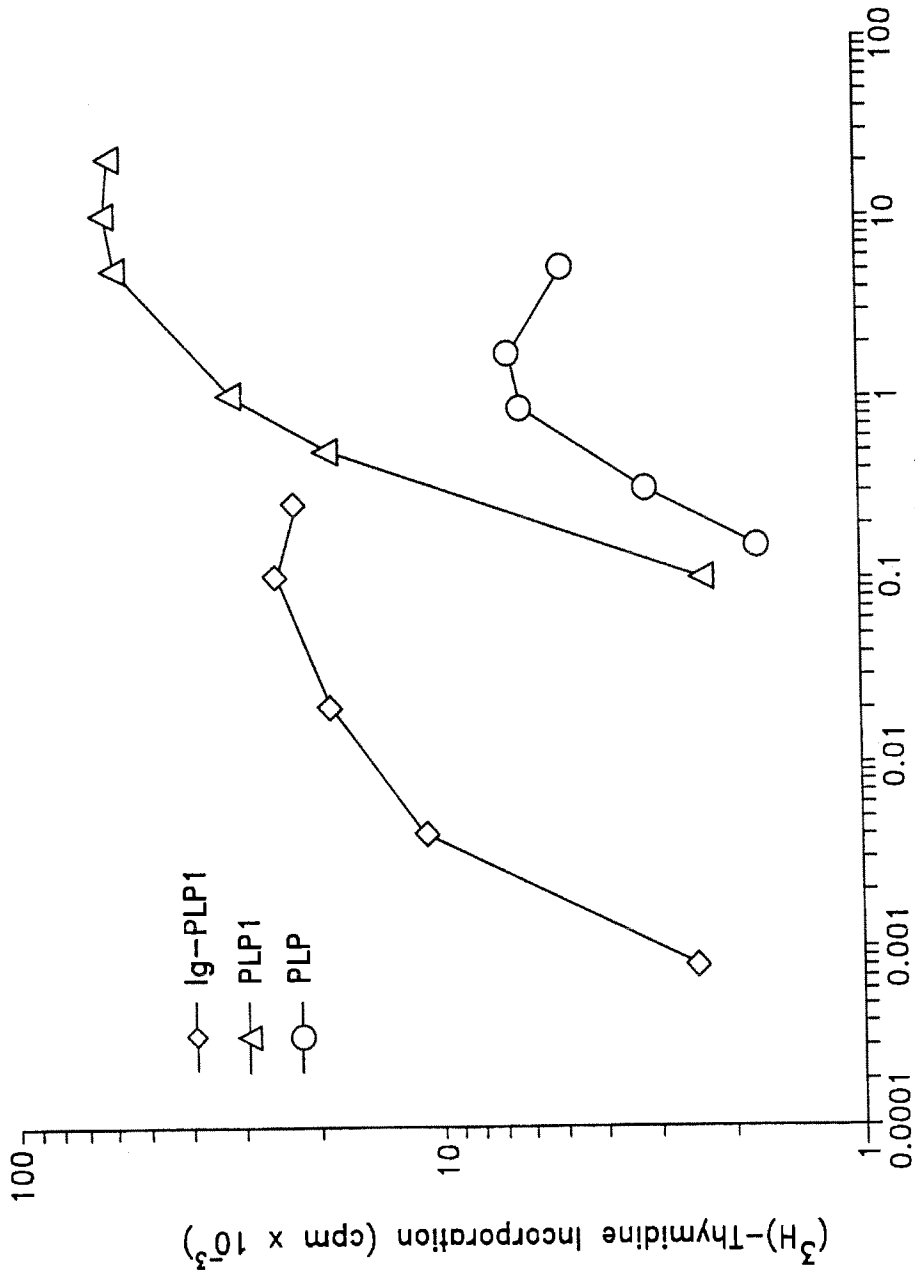

100
10
1
0.0001
0.001
0.01
0.1
1
10
100
Antigen (μM)
Ig-PLP1
PLP1
PLP
(^{3}H)-Thymidine Incorporation (cpm x 10^{-3})

FIG. 4

FIG.5A

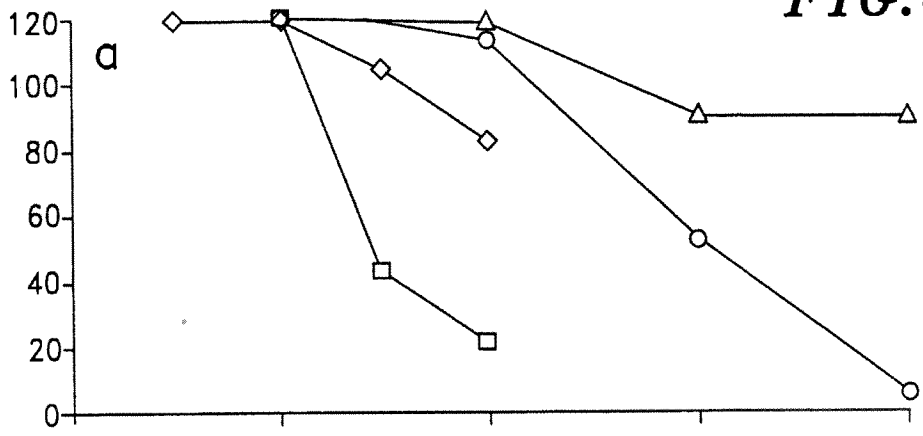


FIG.5B

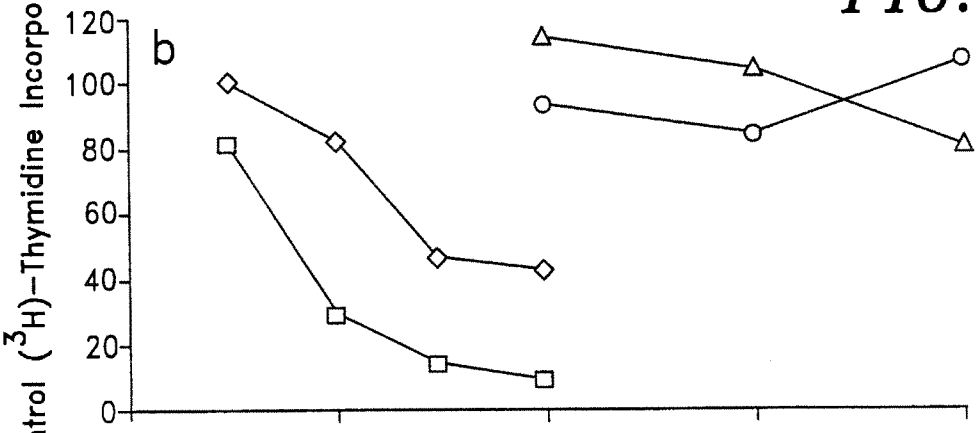
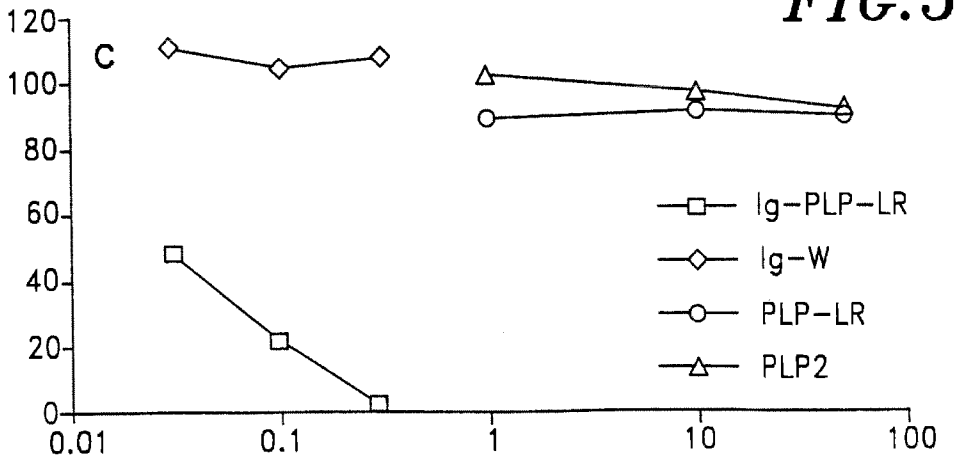


FIG.5C



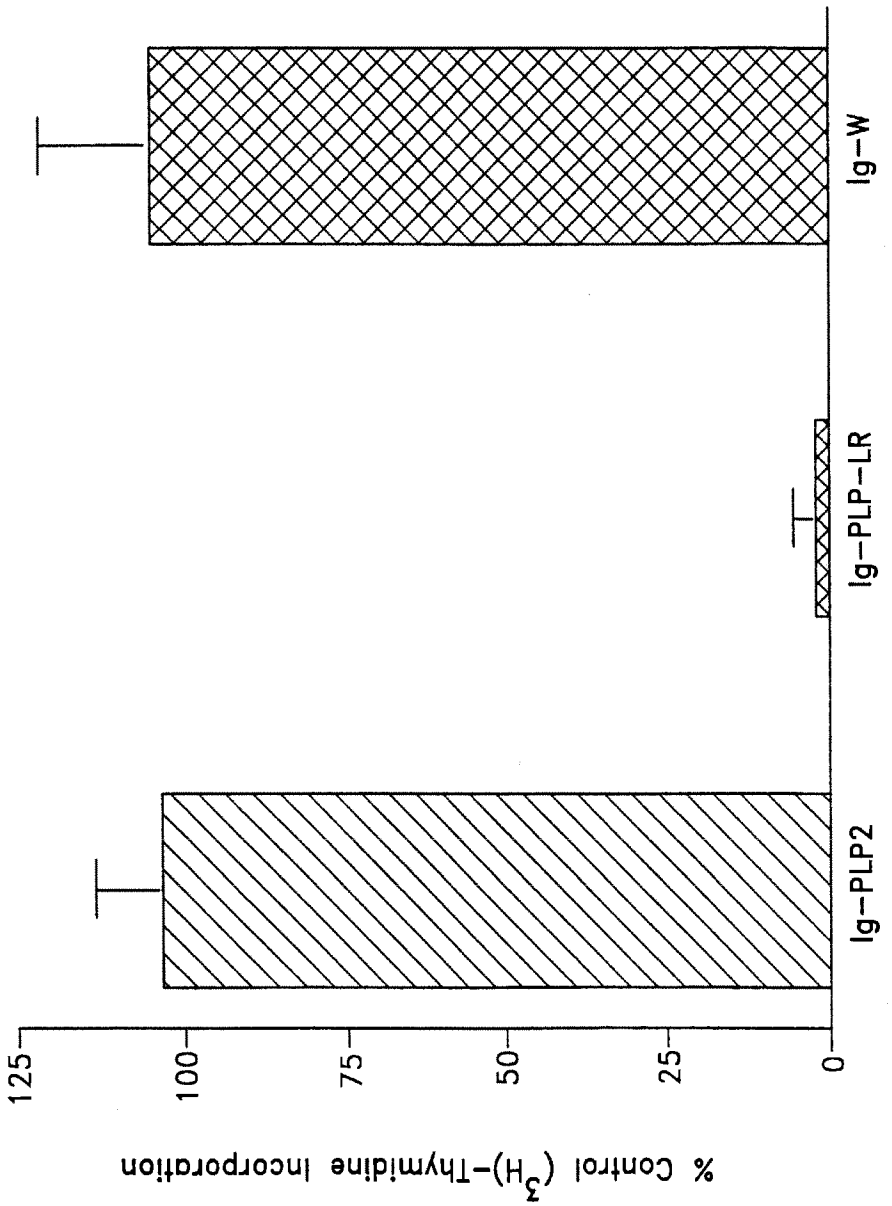

% Control (^{3}H)-Thymidine Incorporation
125
100
75
50
25
0
Ig-PLP2
Ig-PLP-LR
Ig-W

FIG. 6

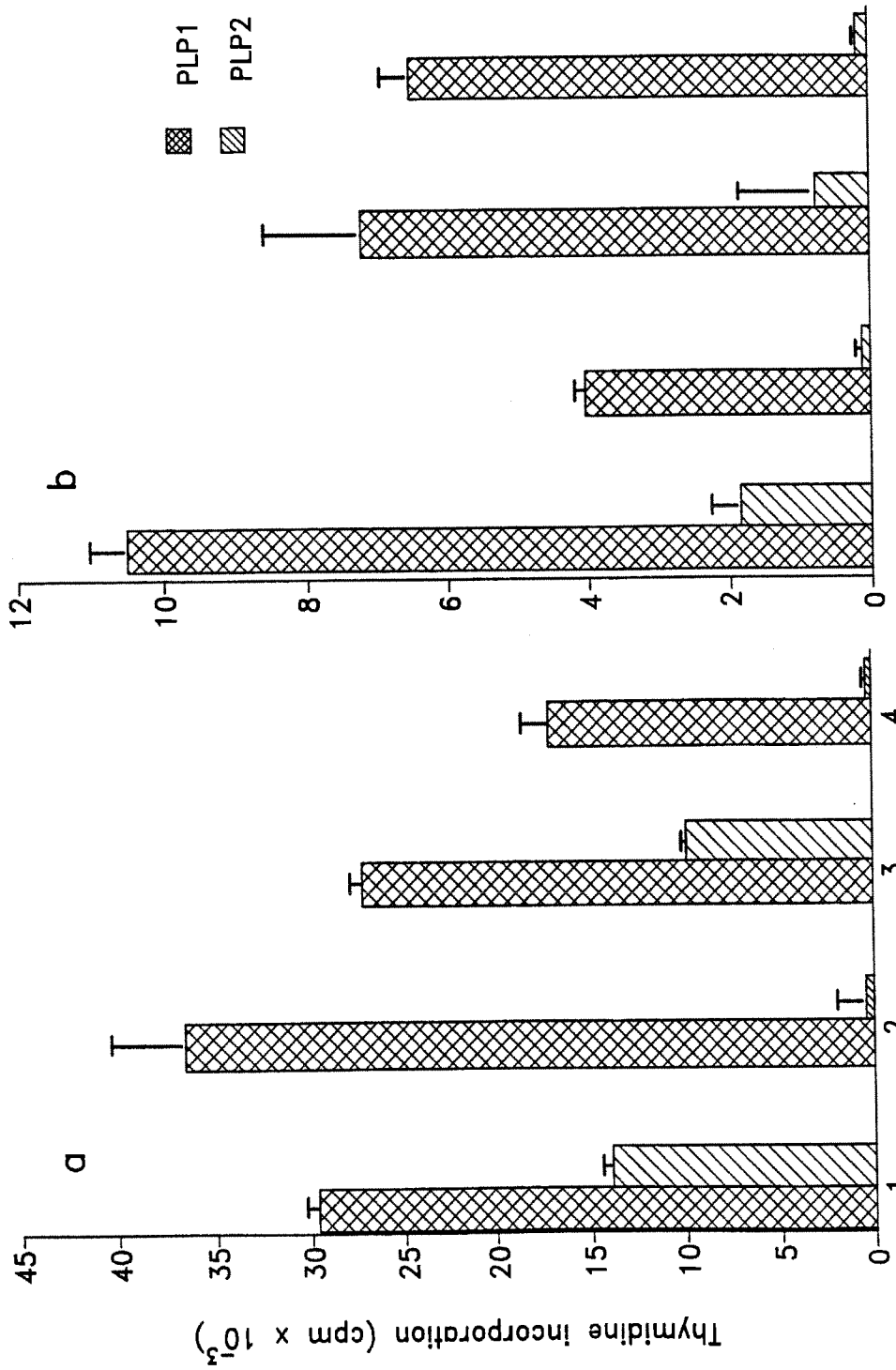

PLP1
PLP2
a
b
Thymidine incorporation (cpm x 10^{-3})
0
5
10
15
20
25
30
35
40
45
0
2
4
6
8
10
12
1
2
3
4

FIG. 7A

FIG. 7B

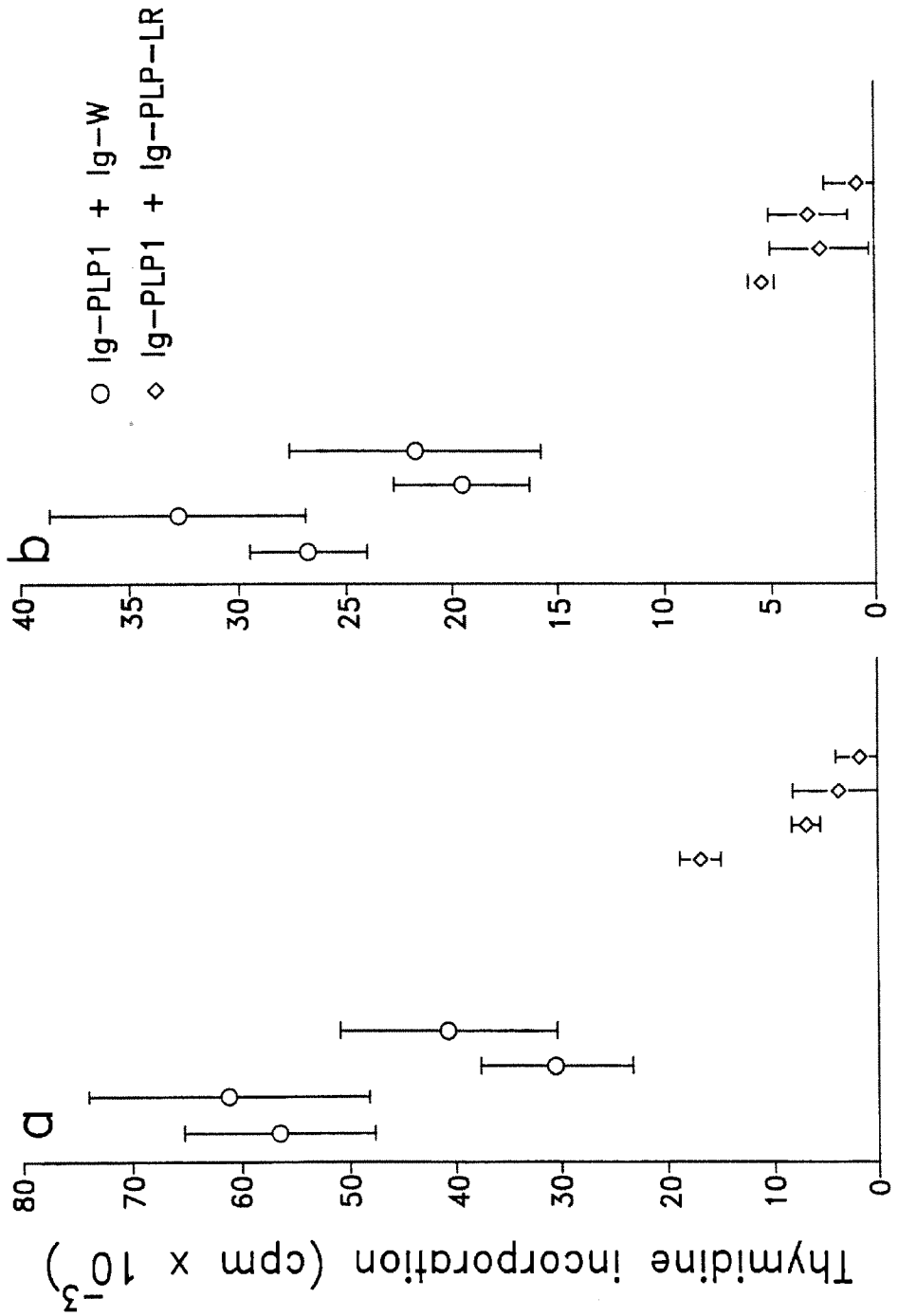

Thymidine incorporation (cpm x 10^{-3})
a
b
○ Ig-PLP1 + Ig-W
◇ Ig-PLP1 + Ig-PLP-LR
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40

FIG.8A

FIG.8B

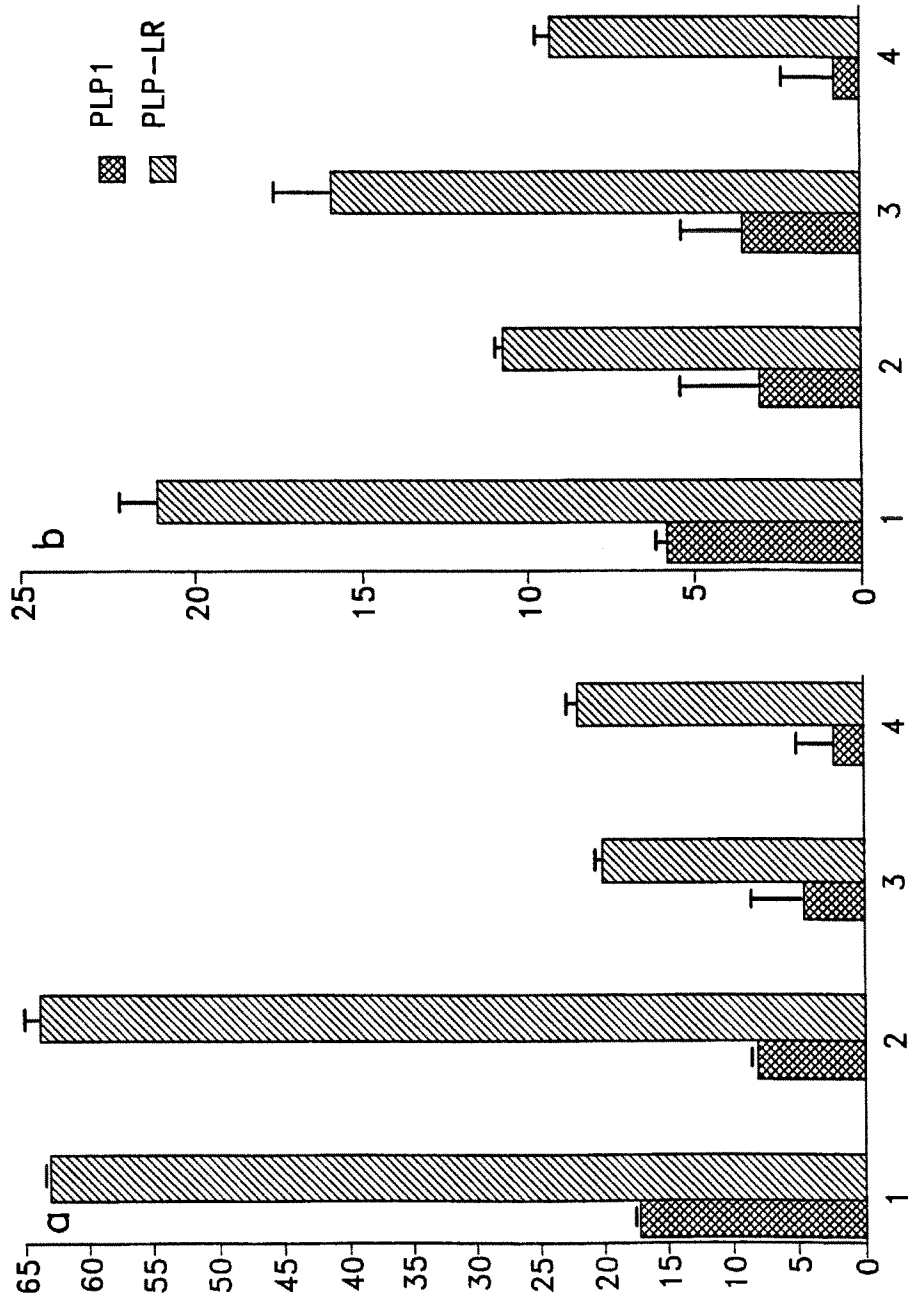

Thymidine incorporation (cpm x 10^{-3})
a
b
PLP1
PLP-LR
0
5
10
15
20
25
30
35
40
45
50
55
60
65
1
2
3
4

FIG. 9A

FIG. 9B

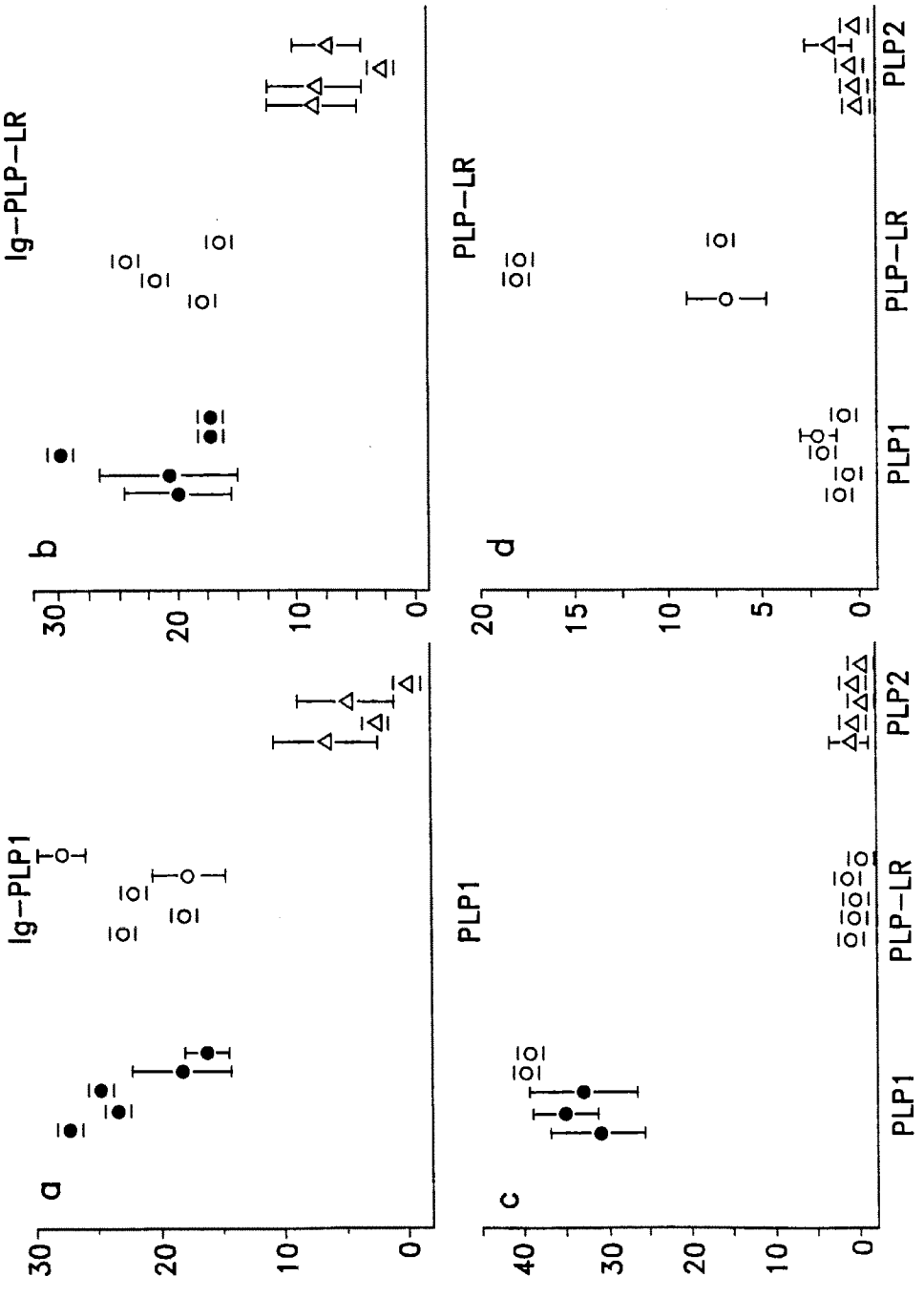

a
Ig-PLP1
PLP1
PLP-LR
PLP2
b
Ig-PLP-LR
c
d
PLP-LR
(3H)-Thymidine incorporation (cpm x 10-3)

FIG. 10

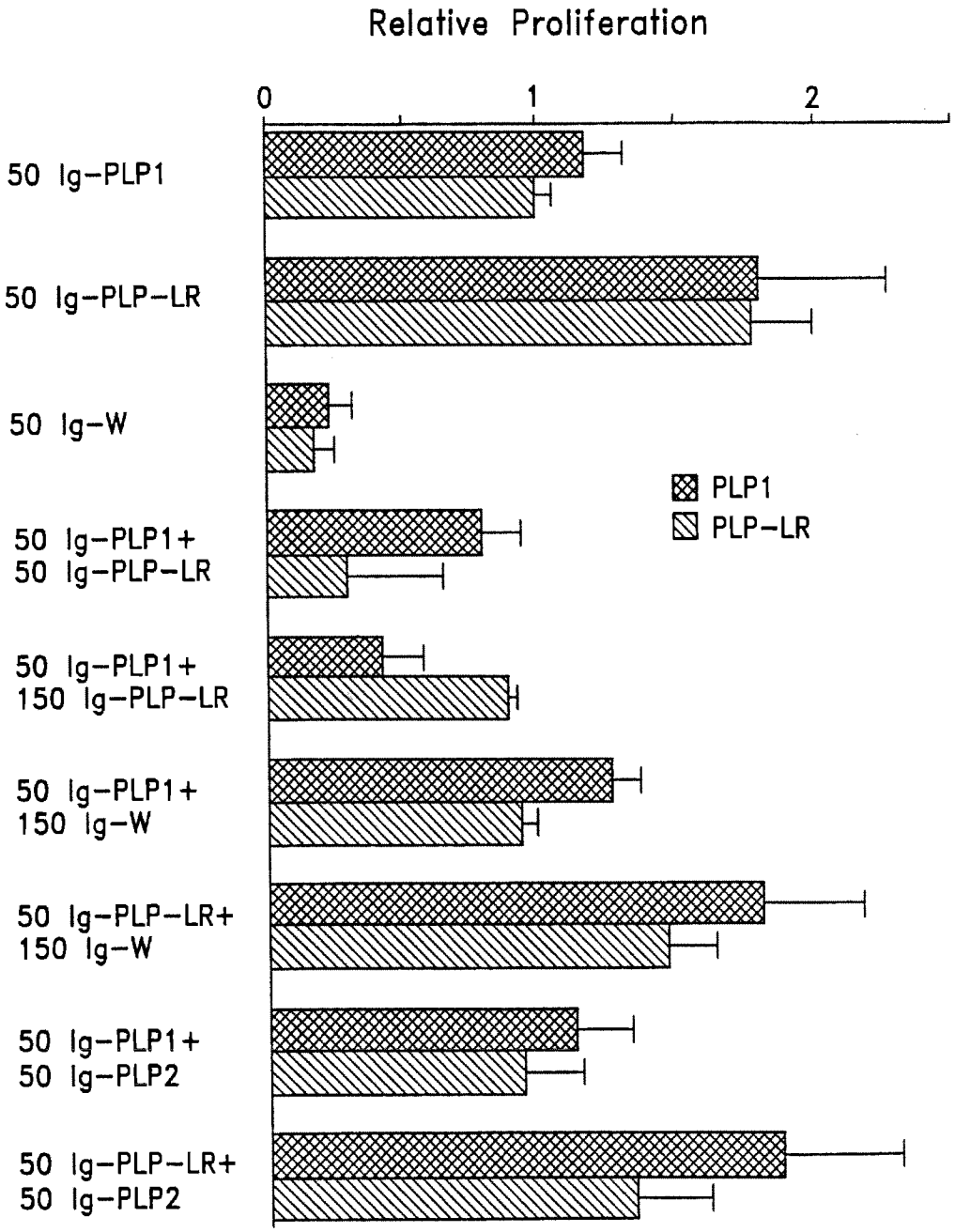

Relative Proliferation
0
1
2
50 Ig-PLP1
50 Ig-PLP-LR
50 Ig-W
50 Ig-PLP1+
50 Ig-PLP-LR
50 Ig-PLP1+
150 Ig-PLP-LR
50 Ig-PLP1+
150 Ig-W
50 Ig-PLP-LR+
150 Ig-W
50 Ig-PLP1+
50 Ig-PLP2
50 Ig-PLP-LR+
50 Ig-PLP2
PLP1
PLP-LR

FIG. 11

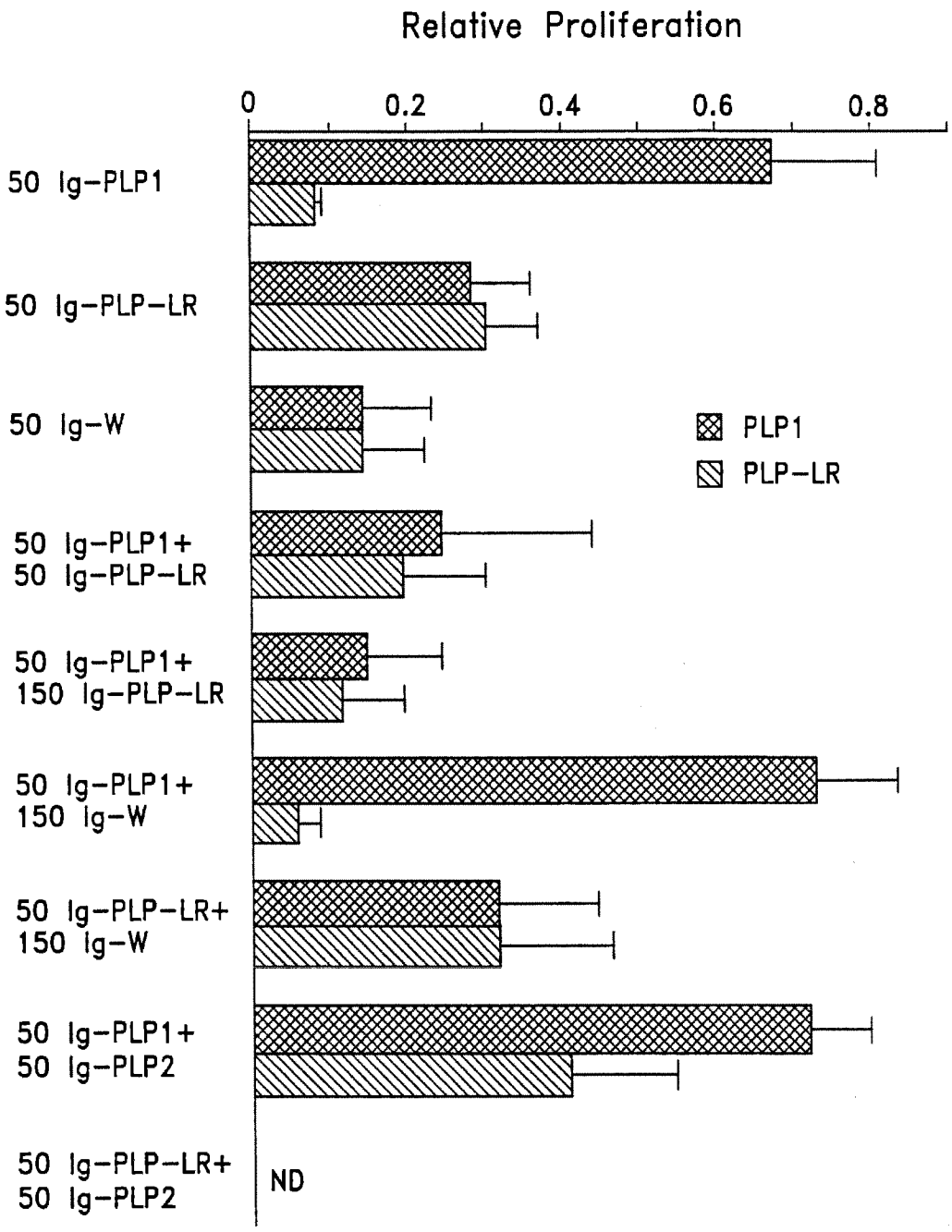

Relative Proliferation
0
0.2
0.4
0.6
0.8
50 Ig-PLP1
50 Ig-PLP-LR
50 Ig-W
50 Ig-PLP1+
50 Ig-PLP-LR
50 Ig-PLP1+
150 Ig-PLP-LR
50 Ig-PLP1+
150 Ig-W
50 Ig-PLP-LR+
150 Ig-W
50 Ig-PLP1+
50 Ig-PLP2
50 Ig-PLP-LR+
50 Ig-PLP2
ND
PLP1
PLP-LR

FIG. 12

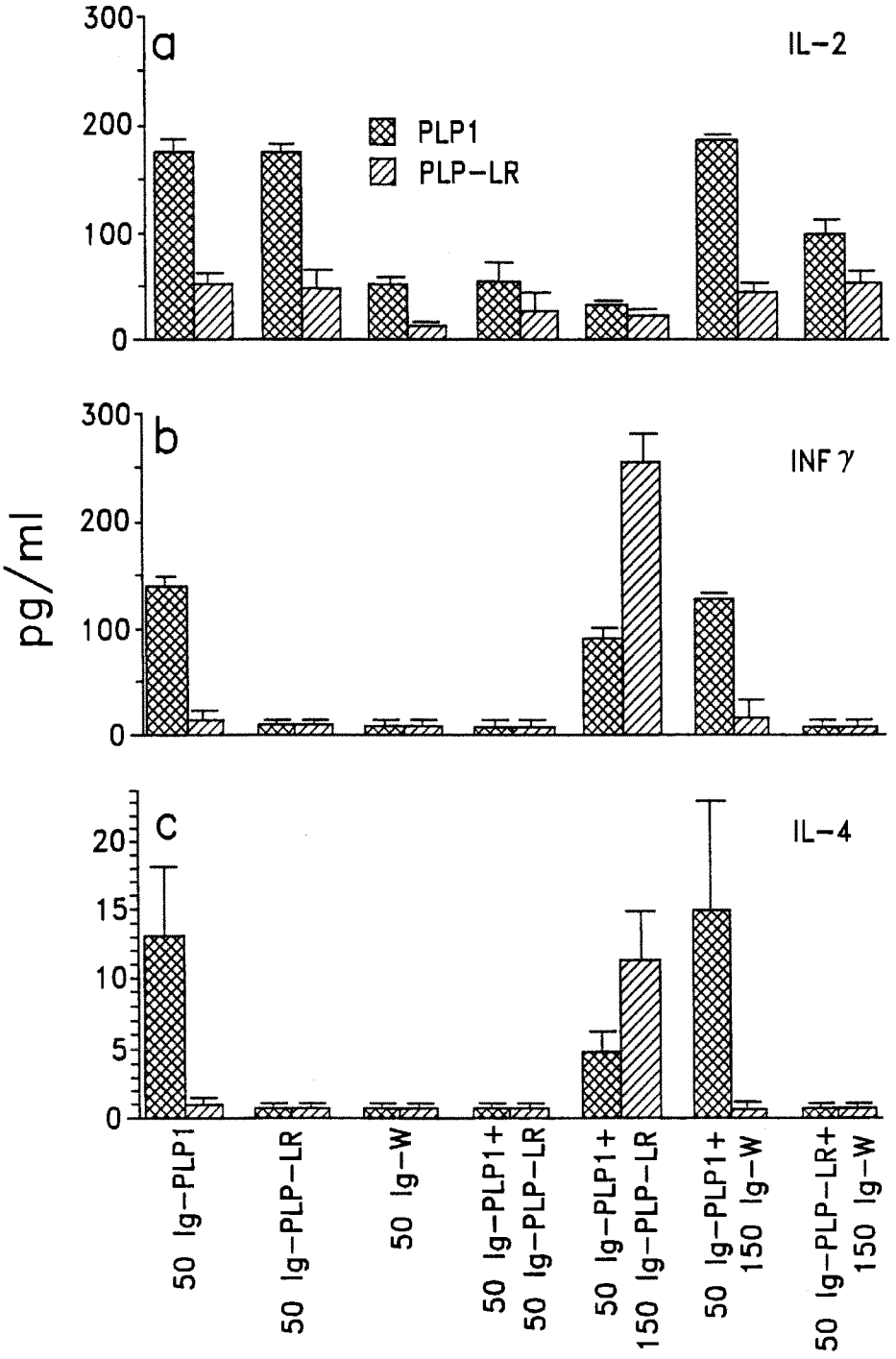

a
IL-2
PLP1
PLP-LR
300
200
100
0
b
INF γ
pg/ml
c
IL-4
20
15
10
5
0
50 Ig-PLP1
50 Ig-PLP-LR
50 Ig-W
50 Ig-PLP1+ 50 Ig-PLP-LR
50 Ig-PLP1+ 150 Ig-PLP-LR
50 Ig-PLP1+ 150 Ig-W
50 Ig-PLP-LR+ 150 Ig-W

FIG. 13

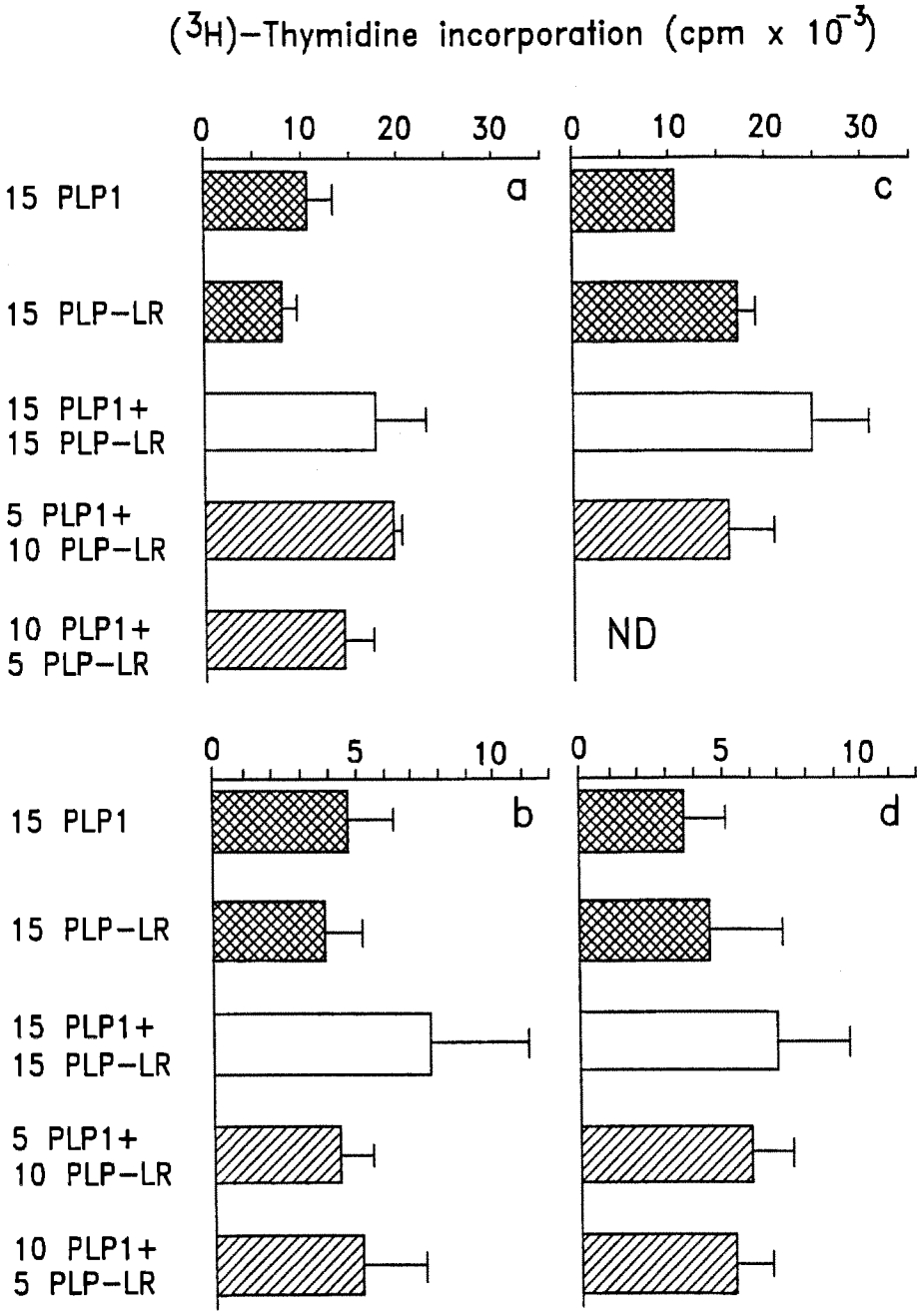

(3H)–Thymidine incorporation (cpm x 10-3)
0
10
20
30
a
c
15 PLP1
15 PLP–LR
15 PLP1+
15 PLP–LR
5 PLP1+
10 PLP–LR
10 PLP1+
5 PLP–LR
ND
0
5
10
b
d
15 PLP1
15 PLP–LR
15 PLP1+
15 PLP–LR
5 PLP1+
10 PLP–LR
10 PLP1+
5 PLP–LR

FIG. 14

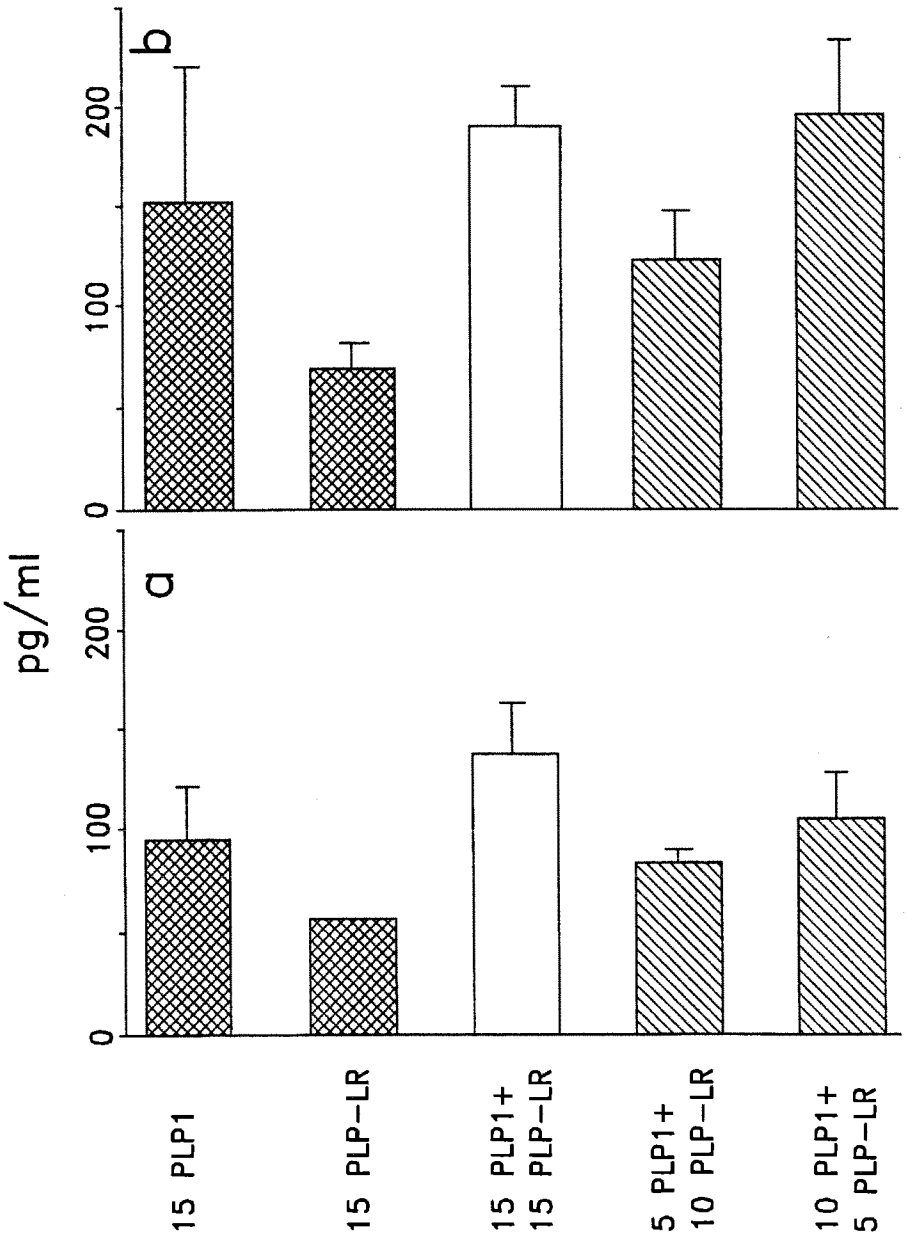

b
a
pg/ml
0
100
200
15 PLP1
15 PLP-LR
15 PLP1+
15 PLP-LR
5 PLP1+
10 PLP-LR
10 PLP1+
5 PLP-LR

FIG. 15

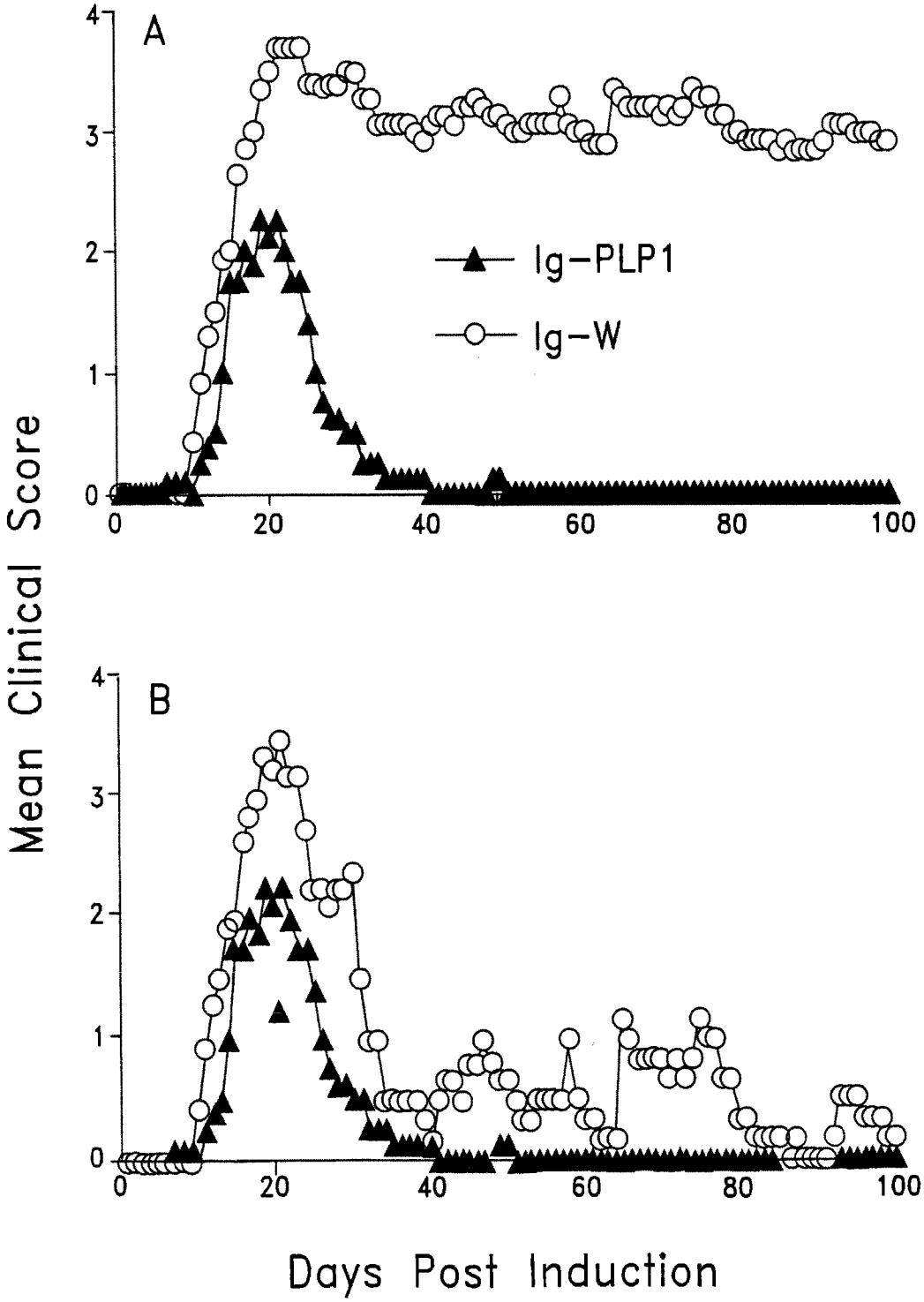

A
B
Ig-PLP1
Ig-W
Mean Clinical Score
Days Post Induction
0
1
2
3
4
0
20
40
60
80
100

FIG. 16

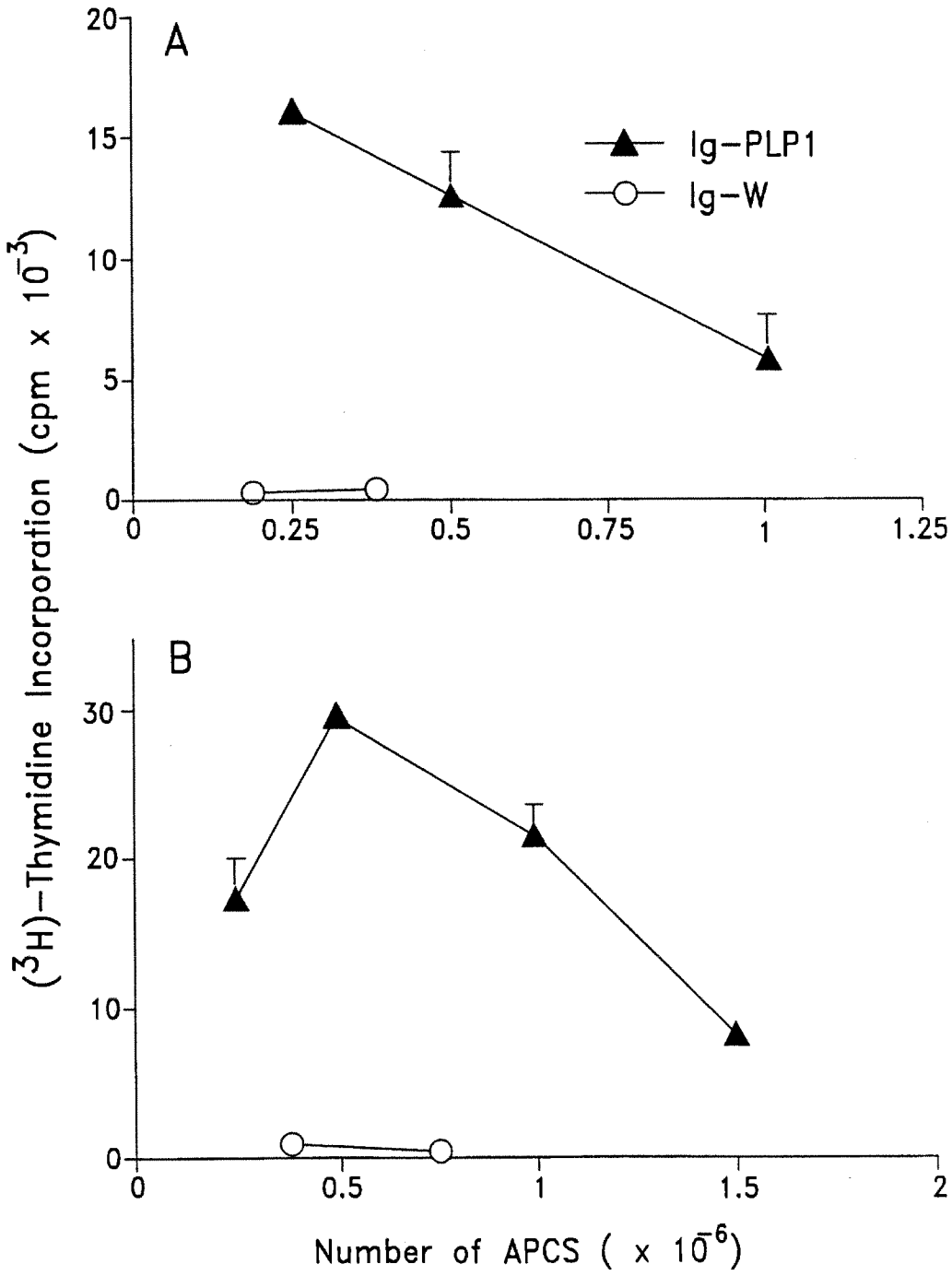

A
B
Ig-PLP1
Ig-W
(3H)-Thymidine Incorporation (cpm x 10-3)
Number of APCS (x 10-6)
0
5
10
15
20
0.25
0.5
0.75
1
1.25
30
1.5
2

FIG. 17

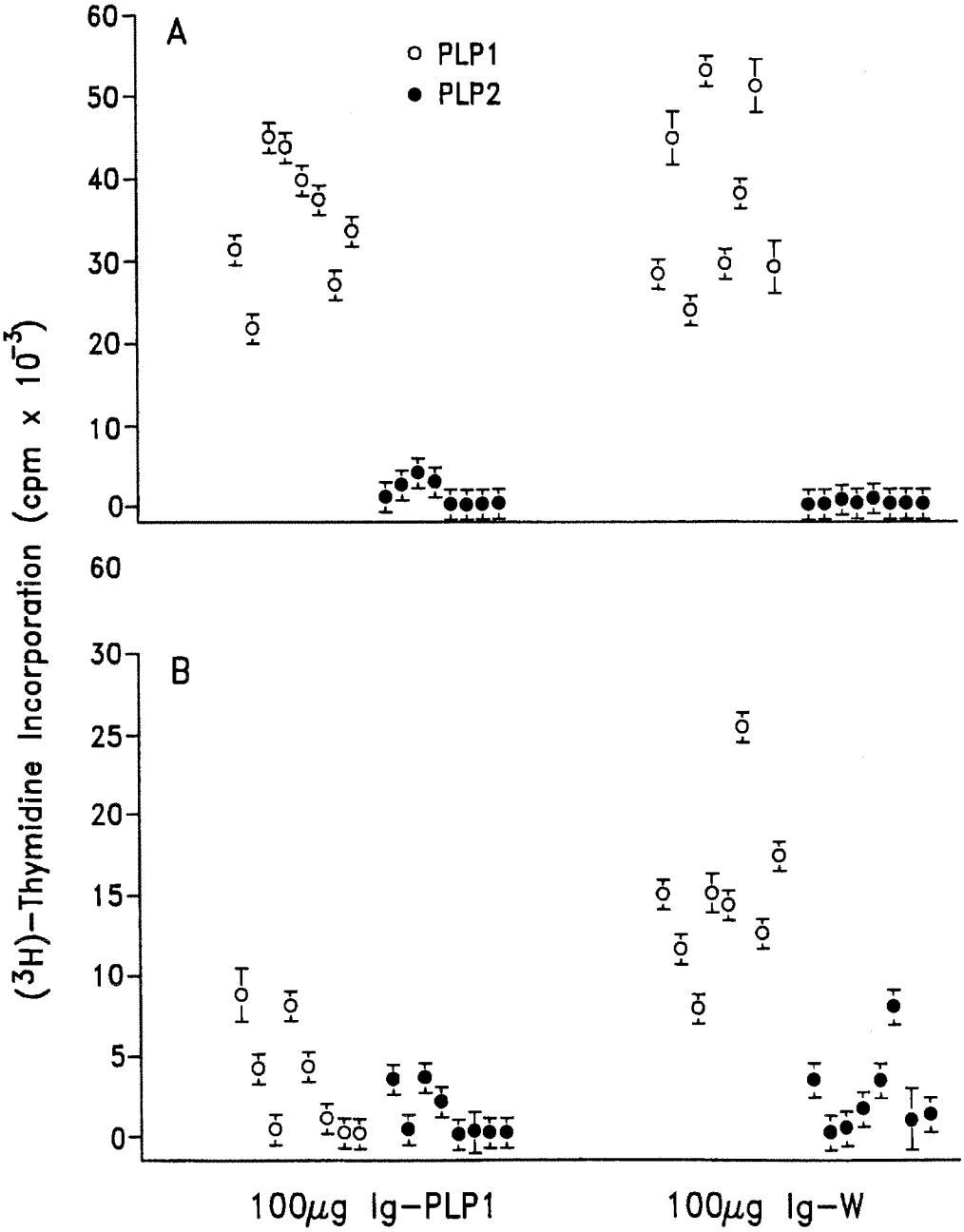

A
B
PLP1
PLP2
(^3H)-Thymidine Incorporation (cpm x 10^{-3})
60
50
40
30
20
10
0
60
30
25
20
15
10
5
0
100μg Ig-PLP1
100μg Ig-W

FIG. 18

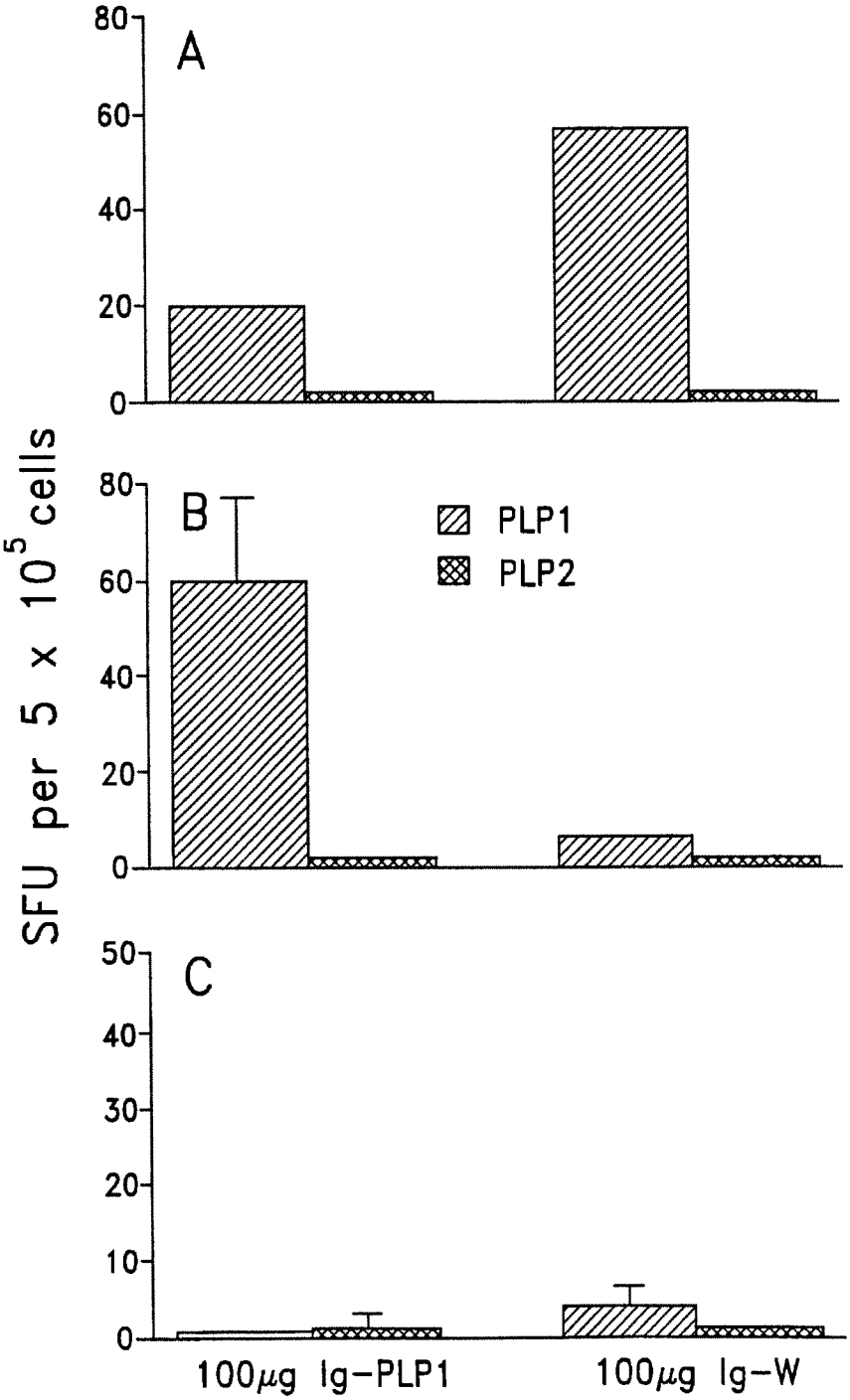

A
B
C
PLP1
PLP2
SFU per 5 x 10^5 cells
80
60
40
20
0
50
30
10
100μg Ig-PLP1
100μg Ig-W

FIG. 19

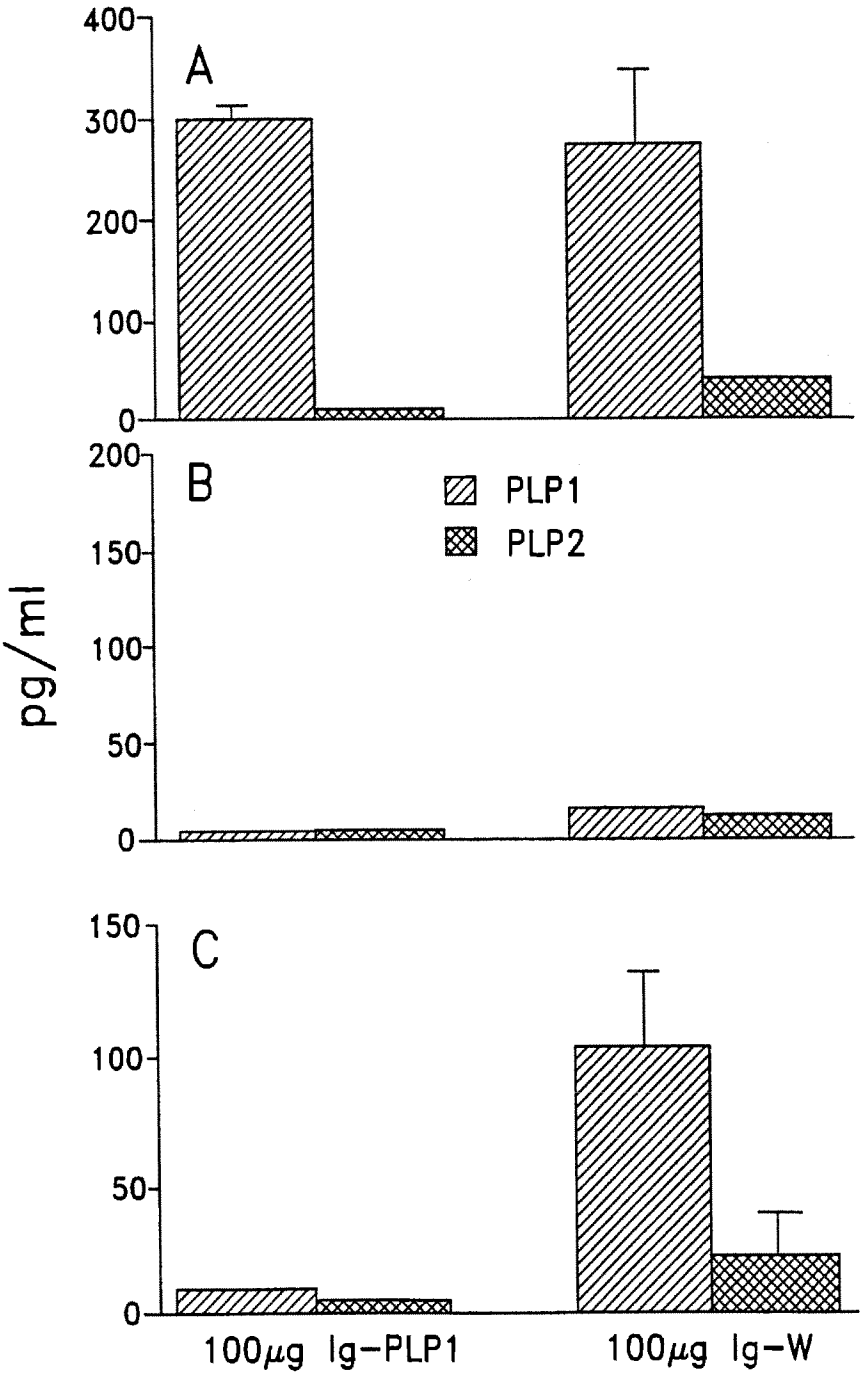

400
300
200
100
0
A
200
150
100
50
0
B
PLP1
PLP2
pg/ml
150
100
50
0
C
100μg Ig-PLP1
100μg Ig-W

FIG.20

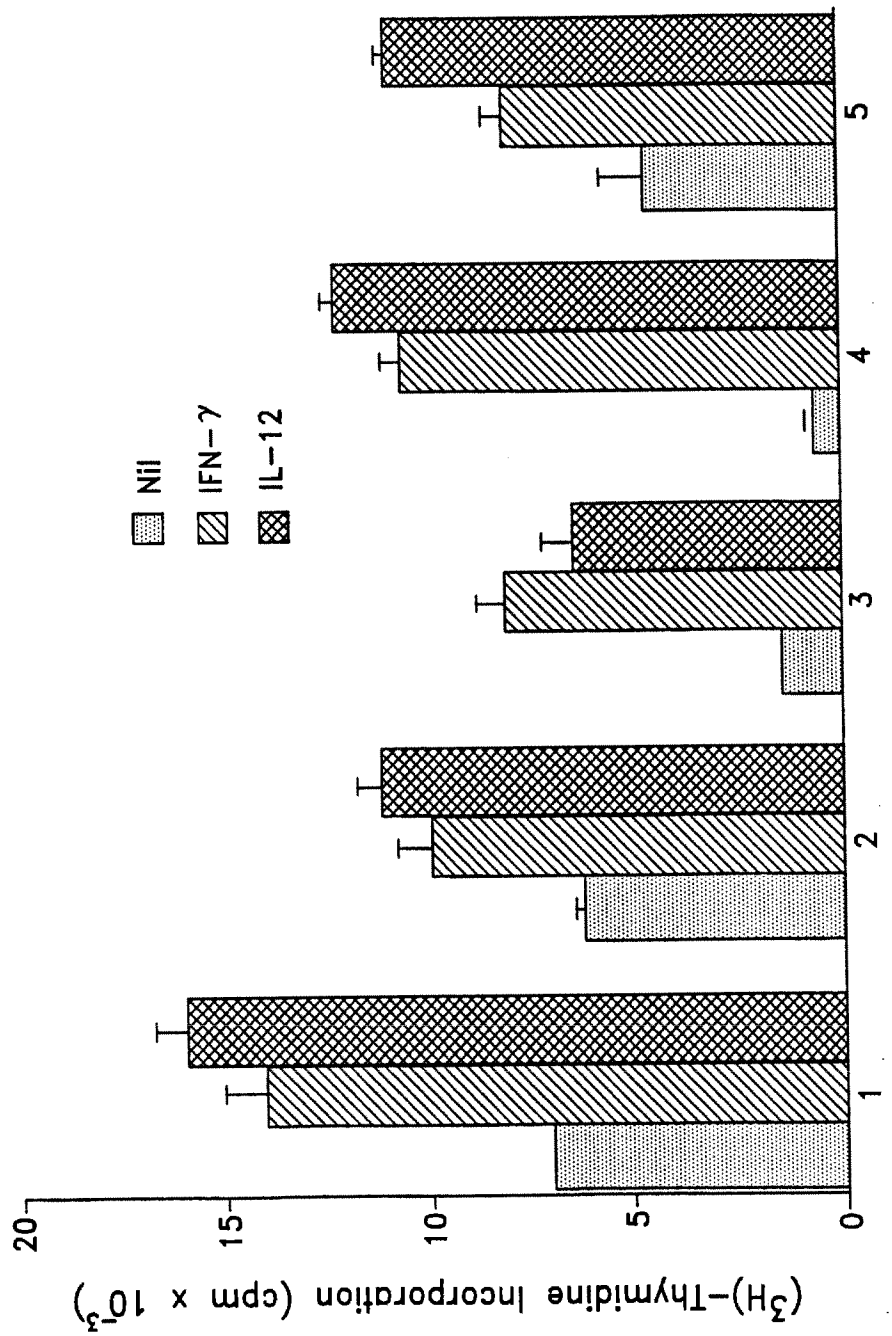

Nil
IFN-γ
IL-12
0
5
10
15
20
1
2
3
4
5
(^{3}H)-Thymidine Incorporation (cpm x 10^{-3})

FIG.21

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 98/00520

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 A61K39/385 C07K19/00 C12N5/10

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MIN ET AL.: "Modulation in vivo of autoreactive T cells by a TCR antagonist Ig Chimera" JOURNAL OF THE ALLERGY AND CLINICAL IMMUNOLOGY, vol. 99, no. 1, January 1997, page S183 XP002065436 * see abstract No. 738 * ---	1-41
X	WO 94 28027 A (ARCH DEVELOPMENT CORP. USA) 8 December 1994 see the whole document --- -/--	1,11-13, 16,19, 23-29, 32,33, 35,38, 40-43

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of theinternational search	Date of mailing of the international search report
19 May 1998	08/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Muller-Thomalla, K

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter: "onal Application No
PC1/US 98/00520

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 629 703 A (MEDAREX) 21 December 1994 see the whole document	1-41
A	WO 92 05793 A (MEDAREX) 16 April 1992 see the whole document	1-41
A	WO 94 10332 A (MEDAREX) 11 May 1994 see the whole document	1-41
A	WO 96 34622 A (ALEXION PHARM INC.) 7 November 1996 see the whole document	1-41
A	WO 93 06135 A (GENENTECH) 1 April 1993 see the whole document	1-41

1

INTERNATIONAL SEARCH REPORT

formation on patent family members

Inter onal Application No
PCT/US 98/00520

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 1500195 T	26-01-89
		JP 2648317 B	27-08-97
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		AU 8869491 A	28-04-92
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		JP 6502410 T	17-03-94
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		EP 0668924 A	30-08-95
WO 9634622 A	07-11-96	AU 5565896 A	21-11-96
		EP 0830139 A	25-03-98
WO 9306135 A	01-04-93	EP 0605606 A	13-07-94
		JP 6511241 T	15-12-94

EXHIBIT C

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9299–9304, August 1999
Immunology

The role of MHC class II molecules in susceptibility to type I diabetes: Identification of peptide epitopes and characterization of the T cell repertoire

CHENG-CHI CHAO[†], HUEY-KANG SYTWU[†‡], EMILY LICHUAN CHEN[†], JON TOMA[†], AND HUGH O. MCDEVITT[†§]

[†]Departments of Microbiology and Immunology, and Medicine, Stanford University Medical Center, Stanford, CA 94305; and [‡]Departments of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan 10050, Republic of China

Contributed by Hugh O. McDevitt, June 2, 1999

ABSTRACT **Susceptibility to type I diabetes is linked to class II MHC alleles in both mouse and man. However, the molecular mechanisms by which MHC molecules mediate disease susceptibility are unknown. To analyze how I-A alleles predispose to, or prevent, the development of type I diabetes, we have chosen, as the first step, to investigate the immune response to an important islet cell protein in diabetes-susceptible and diabetes-resistant mice. MHC class II alleles conferring susceptibility and resistance to diabetes select completely different sets of immunogenic epitopes from the β islet cell autoantigen glutamic acid decarboxylase 65. Peptide-binding studies, analysis of MHC restriction, and immunization with these peptide epitopes indicate that the two amino acid substitutions within the $I\text{-}A_\beta$ chain that distinguish a diabetes-susceptibility from a diabetes-resistance allele are sufficient to alter peptide binding and MHC restriction and may also influence antigen presentation and the selection of the T cell repertoire. The data indicate that the molecular mechanisms for class II-mediated selection of immunodominant epitopes are complex and differ for each individual peptide epitope. Further study of the functional characteristics of the response to these epitopes should provide insight into mechanisms of MHC-mediated diabetes susceptibility.**

Type I diabetes and several other autoimmune diseases, including rheumatoid arthritis, show strong association and linkage with very specific sequence polymorphisms in MHC class II molecules (1). These polymorphisms are found in several different, susceptible MHC alleles in humans and animals. In nonobese diabetic (NOD) mice, the MHC class II region encodes a unique I-A molecule ($I\text{-}A^{g7}$) and a nonexpressed I-E molecule (2, 3). The $I\text{-}A^{g7}_\beta$ allele in NOD mice encodes serine instead of aspartic acid at position 57. Transgenic introduction of a resistant $I\text{-}A_\beta$ chain, or a mutated $I\text{-}A^{g7}$ β chain, converts the susceptible into a resistant phenotype. Several groups have produced NOD mice that are transgenic for a "nonsusceptible" (aspartic acid at position 57) MHC class II I-A allele. These I-A transgenic (Tg) mice on the NOD background exhibit markedly decreased levels of insulitis and diabetes (4–6). More specifically, Lund and co-workers (7) have shown that transgenic NOD mice carrying an $I\text{-}A^{g7}$ allele mutated at position 56 (His to Pro) or 57 (Ser to Asp) are partially or completely protected from both diabetes and insulitis. These results show that $I\text{-}A_\beta$ position 57 is the major MHC-linked genetic polymorphism determining susceptibility or resistance to type I diabetes.

Although associations and linkage of MHC class I and II genotype with disease susceptibility have been shown in several autoimmune diseases, the mechanisms of susceptibility are not understood. Two T cell receptor (TCR) transgenic models recently have been used to investigate the molecular basis for resistance to type I diabetes. One (8) found that protective MHC class II molecules provide resistance via thymic deletion of diabetogenic T cells. The second study (9) found that resistant class II alleles cause positive selection of regulatory T cells. Both results may be correct, but only for their respective TCRs.

A third study (10) found that one diabetogenic TCR (see ref. 9) was positively selected by diabetes-susceptible ($I\text{-}A^{g7}$) as well as diabetes-resistant ($I\text{-}A^{g7/2Cr}$) class II alleles, and neither allele negatively selected this TCR in the periphery. Again, these results apply only to this TCR.

In any event, these studies investigate protection from diabetes induced by a single transgenic TCR, but do not address how susceptible class II alleles mediate susceptibility. To understand susceptibility, it may be necessary to characterize completely the autoimmune response in type I diabetes. This will require identifying the critical target autoantigens for type I diabetes, their peptide epitopes, and the functional characteristics [e.g., T helper 1 (Th1) vs. Th2] of responding T cells. Do susceptible and resistant alleles select functionally different T cell repertoires specific for autoantigen epitopes? What are the effects of susceptible and resistant alleles on antigen processing/presentation, interaction with CLIP and H-2M molecules, and on proteolytic processing pathways?

To initiate this approach, we designed alternative experiments to investigate the molecular mechanisms of MHC-linked disease susceptibility and protection. NOD wild-type and NOD.PD Tg mice were used as animal models for type I diabetes-susceptible and diabetes-resistant MHC genotypes, respectively. NOD.PD Tg mice express a site-specific mutant $I\text{-}A^{g7}_\beta$ allele in which amino acids 56 and 57 have been mutated from histidine and serine to proline and aspartic acid (11). The only difference between wild-type NOD mice and NOD.PD mice is at positions 56 and 57 of the transgenic MHC class II $I\text{-}A_\beta$ chain. NOD.PD Tg mice have been fully backcrossed to NOD mice, and these mice do not develop diabetes.

The first step in this experimental approach is to characterize the immunogenic T cell epitopes of islet cell autoantigens in diabetes-susceptible and diabetes-resistant strains. The islet cell autoantigen chosen in this study is glutamic acid decarboxylase 65 (GAD65), which has been shown to play an important role in the pathogenesis of type I diabetes in humans and in NOD mice (12–15). After identification of the immunogenic epitopes of GAD65, possible mechanisms for control of MHC-linked susceptibility/resistance to diabetes were analyzed by studying MHC restriction, peptide-binding affinity, cytokine response profiles,

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Abbreviations: GAD65, glutamic acid decarboxylase 65; TCR, T cell receptor; APC, antigen presentation cells; Tg, transgenic; Th, T helper; IFA, incomplete Freund's adjuvant; NOD mice, nonobese diabetic mice.

[§]To whom reprint requests should be addressed at: Department of Microbiology and Immunology, Sherman Fairchild Building, Room D345, Stanford University, Stanford, CA 94305. E-mail: HughMcD@leland.stanford.edu.

and T cell responses to immunization with peptides identified in susceptible and resistant mice.

The results show that I-A^{g7} molecules select a completely different set of immunogenic epitopes of GAD65 for presentation to CD4 T cells from those identified by the diabetes-resistant I-$A^{g7.PD}$ molecules. The two amino acid substitutions within the I-A_β chain that distinguish NOD wild-type mice from NOD.PD Tg mice are sufficient to influence dramatically MHC restriction, peptide binding, selection of the T cell repertoire and/or antigen-presentation pathways. Previous studies suggested that protective class II alleles mediated their effects either by positive selection of regulatory T cells (9) or by thymic deletion of autoreactive T cells (8). The data in the present study are compatible with these previous results and provide libraries of peptide epitopes and peptide-specific T cell hybridomas, which can be used to further analyze mechanisms of MHC-mediated susceptibility to type I diabetes.

MATERIALS AND METHODS

Generation and Screening of NOD.PD Tg Mice. The construction of the NOD.PD transgene and characterization of the resulting Tg mouse lines have been described in detail elsewhere (11). All Tg mice in this study were heterozygous for the NOD.PD transgene. Outcrosses to BALB/c indicate that I-$A^{g7.PD}$ is expressed at a level 100–150% that of I-A^{g7}.

To identify mice carrying the NOD.PD transgene, three oligonucleotide primers spanning the mutated region were designed to screen mice in two separate PCRs. Primer EC-PD4 (5′-CACCAGTTCAAGGGCGAG-3′), which annealed upstream of the mutated site, served as the 5′ primer. Two 3′ primers were created. Primer EC-PD5 (5′-TTGTAGTACTCGGCGTCC-3′) includes a 4-bp sequence discrepancy at the 3′ end that anneals only to the PD transgene but not to the g7 allele, when the proper annealing temperature is used. Only DNA from NOD.PD Tg mice will generate a 161-bp DNA product with primers EC-PD4 and EC-PD5 (annealing temperature 56°C). Primer EC-PD6 (5′-CCGCAGGGAGGTGGGGAC-3′) anneals downstream from EC-PD5. PCRs using EC-PD4 and EC-PD6 (annealing temperature 58°C) will yield a 255-bp PCR product in both Tg and non-Tg mice. The sequence difference between the PD transgene and I-A^{g7} creates an *Eag*I site in the PD transgene. Digestion of the 255-bp product from the PD transgene by *Eag*I yields 139- and 116-bp fragments, whereas the 255-bp product from the g7 template remains undigested.

Generation of GAD65-Specific T Cell Hybridomas. Nine-week-old female NOD (The Jackson Laboratory) and NOD.PD Tg mice were immunized in the hind footpads and at the base of the tail with 50 μg of GAD65 protein in incomplete Freund's adjuvant (IFA). Ten days later, cells were isolated from popliteal and inguinal lymph nodes, resuspended at 5×10^6 cells per ml in RPMI complete medium containing 1% NOD mouse serum, and restimulated *in vitro* with 10 μg/ml GAD65. Four days later, the cells were purified by Lympholyte-M (Cedarland Laboratories, Ontario, Canada) separation and cultured with 10 units/ml rIL-2 overnight. The GAD65-activated T cells were fused with the BW 5147 cell line by using 50% polyethylene glycol (Sigma). A detailed protocol for generation of T cell hybridomas has been described elsewhere (16).

Epitope Screening Using a Europium-Based IL-2 Sandwich ELISA. The epitope mapping of GAD65 involved three consecutive steps (16). T cell hybridomas initially were screened with GAD65 whole protein. The hybridomas that responded to GAD65 subsequently were screened with 10 pools (10–12 peptides in each pool) of 15-mer peptides overlapping by 10 aa, spanning the entire 585-aa sequence to identify the peptide(s) encoding GAD65 epitopes. The epitope specificity of pool-positive hybridomas was decoded by using individual peptides from the regional pools.

A culture condition for screening GAD65-specific T cell hybridomas has been described elsewhere (16). The ability of the hybridomas to respond to antigen (peptide or protein) was assessed by IL-2 production, as detected by sandwich ELISA.

Competitive Inhibition Peptide–MHC Binding Assay. The peptide–MHC-binding assay used in this study was modified from the protocol originally developed by Nepom and coworkers (17). M12.C3.g7 and M12.C3.g7.PD B cell transfectants express exclusively I-A^{g7} and I-$A^{g7.PD}$ molecules, respectively. These two cell lines had been subcloned and sorted by fluorescent cell sorter to obtain the same levels of surface expression of I-A^{g7} and I-$A^{g7.PD}$ molecules. M12.C3.g7 and M12.C3.PD transfectants were fixed with 0.5% paraformaldehyde. The cells then were washed and the cell pellets were resuspended in 200 μl of citrate phosphate binding buffer, pH 4.5. Various concentrations of unlabeled inhibitory peptides then were added to the cell suspensions. After 4 hr of incubation at 37°C, a biotinylated reference peptide was added and the mixture was incubated for 18–24 hr at 37°C. The biotinylated peptides used as reference peptides were known to bind well to their target MHC molecules (18). The reference peptide for binding to M12.C3.g7 (I-A^{g7}) transfectants was λ repressor 12–24 peptide. The reference peptide for binding to M12.C3.g7.PD (I-$A^{g7.PD}$) transfectants was ovalbumin 323–339 peptide. After incubation overnight, the cells were washed with Hanks' balanced salt solution and lysed with 100 μl of 1% Nonidet P-40 with protease inhibitors. The cell lysates were transferred to ELISA plates precoated with I-A^{g7}-specific mAb (OX-6) (PharMingen) and incubated overnight at 4°C. After washing, europium-labeled streptavidin was added at 100 μl per well and incubated for 1 hr at room temperature. The level of fluorescence was measured with an LKB-Wallac fluorescence plate reader (Wallac, Gaithersburg, MD).

Measurement of Antigen-Specific Cytokine Expression by ELISA. Wild-type NOD and NOD.PD mice were immunized with 50 μg of GAD65 protein in IFA in the hind footpads and the base of the tail. Ten days later, draining lymph node cells were isolated and stimulated *in vitro* with 20 μg/ml GAD65, a "g7" or "PD" peptide pool, or individual "g7" or "PD" peptides (20 μM/peptide) for 72 hr. Culture supernatants were harvested, and cytokine (IFN-γ and IL-4) production was determined by immunoassay by using purified capture and biotinylated detection monoclonal pairs (PharMingen).

RESULTS

Mapping of Immunogenic T Cell Epitopes of GAD65 in NOD Wild-Type Mice and Characterization of the Core Sequences of These Epitopes. Nine-week-old NOD mice were immunized with GAD65 protein in IFA to generate T cell hybridomas for epitope mapping. The immunogenic epitopes of GAD65 identified in NOD mice and the frequency of GAD65 peptide-specific T cell hybridomas are shown in Table 1. Five immunogenic epitopes (p206–220, p221–235, p286–300, p401–415, and p561–575) were identified. p206–220, p221–235, and p286–300 are the three most frequent epitopes among the five identified. Forty percent of GAD65-specific T cell hybridomas recognize p206–220, and 39% respond to p221–235. Unimmunized NOD mice 9 weeks of age also were used to perform epitope mapping. In 780 screened T cell hybridomas, two and one of the hybridomas were specific for p286–300 and p206–220, respectively. None of the epitopes identified in this study overlap with the three GAD65 epitopes

Table 1. Immunogenic T cell epitopes of GAD65 identified in NOD mice

Peptide	No. of T cell hybridomas	% (n = 74)
206–220	30	41
221–235	29	39
286–300	7	9
401–415	3	4
561–575	5	7

(p247, p509, and p524) previously described by Kaufman *et al.* (13).

Fine mapping of peptide epitopes identified in NOD mice was performed by using variant peptides that were truncated gradually from the N and C termini of the epitopes. Three p206–220-specific and three p286–300-specific T cell hybridomas were used for characterizing the core sequences of their respective peptide epitopes. Five p221–235-specific T cell hybridomas were used for identifying their core sequence. The same core epitope was identified for all the hybridomas of a given specificity. The length of the peptide core of p206–220 and p221–235 needed for optimal T cell response is 10 residues, defined as 208–217 (Fig. 1) and 223–232 (data not shown). The peptide core for p286–300 is shorter, and a seven-residue peptide (p289–295) is able to induce a T cell response (data not shown).

Mapping of Immunogenic T Cell Epitopes of GAD65 in NOD.PD Tg Mice and Identification of MHC Restriction of These T Cell Hybridomas. The generation and screening of T cell hybridomas from NOD.PD mice (diabetes-resistant strain) were accomplished by using a protocol identical to that described in the previous section. Nine-week-old NOD.PD mice were immunized with GAD65 in IFA to generate GAD65-specific T cell hybridomas. Table 2 shows the number and frequency of GAD65-specific T cell hybridomas generated from NOD.PD mice. Eight immunogenic epitopes of GAD65 were identified in NOD.PD mice. Five of these T cell epitopes had been found previously in wild-type NOD mice. However, three of the epitopes (p456–470, p331–345, and p551–565) are newly identified epitopes. P456–470 is the dominant epitope of GAD65 derived from NOD.PD mice. Fifty-two percent of GAD65-specific T cell hybridomas derived from NOD.PD mice responded to p456–470.

Because endogenous I-A^{g7} molecules also are expressed on the surface of antigen presentation cells (APC) in NOD.PD Tg mice, it is essential to determine the MHC restriction of the T cell hybridomas derived from NOD.PD mice. To determine MHC restriction, we tested the response of individual T cell hybridomas to a specific peptide presented by M12.C3.g7 or M12.C3.g7.PD B cell transfectants. M12.C3.g7 and M12.C3.g7.PD transfectants express exclusively I-A^{g7} and I-$A^{g7.PD}$ molecules, respectively. If T cell hybridomas responded more strongly to their specific antigen presented by I-A^{g7} or I-$A^{g7.PD}$ molecules, these T cell hybridomas were considered to be restricted to I-A^{g7} or to I-$A^{g7.PD}$ molecules. The results of these experiments with hybridomas from NOD.PD mice are shown in Fig. 2. For the five epitopes, p206–220, p221–235, p286–300, p401–415, and p561–575, the T cell hybridomas gave a much stronger response to peptide plus I-A^{g7} and gave a weak or no response to peptide plus I-$A^{g7.PD}$. We therefore refer to these five I-A^{g7}-restricted T cell epitopes as "g7" epitopes. For the other three epitopes (p456–470, p331–345, and p551–565), T cell hybridomas had much stronger responses to peptide plus I-$A^{g7.PD}$, but not I-A^{g7} molecules. These three I-$A^{g7.PD}$-restricted T cell epitopes therefore are designated as "PD" epitopes. Several T cell hybridomas with the same peptide specificity were tested for MHC restriction and uniformly gave the same pattern for all eight epitopes. The degree of responses to p221–235 showed some extent of variation between tested T cell hybridomas. However, these p221–235-specific hybridomas consistently gave a stronger response to the peptide plus I-A^{g7} than I-$A^{g7.PD}$. These g7-restricted and PD-restricted T cell epitopes of GAD65, with their amino acid sequences, are listed in Table 3.

Table 2. Immunogenic T cell epitopes of GAD65 identified in NOD.PD transgenic mice

Peptide	No. of T cell hybridomas	% (n = 81)
456–470	42	52
331–345	5	6
551–565	8	9
206–220	3	4
221–235	2	2
286–300	11	13
401–415	3	4
561–575	7	8

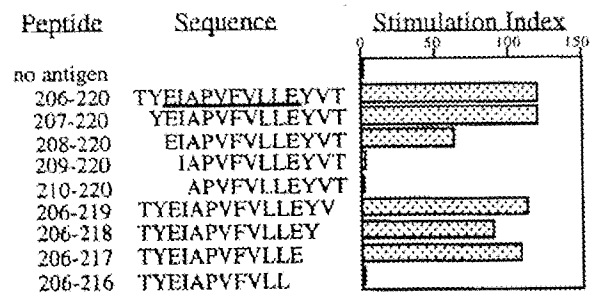

FIG. 1. Identification of the peptide core sequence to p206–220-specific T cell hybridomas. T cell hybridomas were stimulated with 5 μg/ml wild-type or variant peptides for 48 hr. The ability of the hybridomas to respond to antigen was assessed on the basis of the level of IL-2 production, as detected by ELISA. The stimulation index is the ratio of IL-2 level from culture supernatants with peptides to that of culture supernatants without peptides. The underlined sequence represents the peptide core sequence of that epitope.

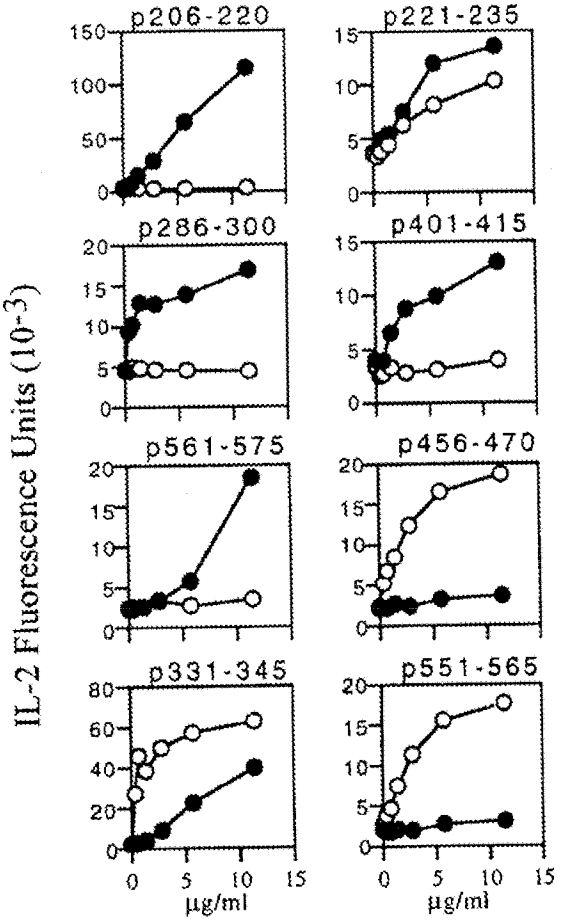

FIG. 2. MHC restriction of GAD65-specific T cell hybridomas. T cell hybridomas were stimulated with various concentrations of specific peptides presented by M12.C3.g7 (●) or M12.C3.g7.PD (○) cells for 48 hr. The ability of the hybridomas to respond to antigen was assessed on the basis of the level of IL-2 production. The x axis represents the concentration of peptide, and the y axis represents the arbitrary fluorescence units obtained from the IL-2 ELISA.

Table 3. g7 and PD epitopes and their amino acid sequences

Epitope	Peptide	Sequence
g7	206–220	TYELAPVFVLLEYVT
	221–235	LKKMREHGWPGGSG
	286–300	KKGAAAIGIGTDSVI
	401–415	PLQCSALLVREEGLM
	561–575	ISNPAATHQDIDFLI
PD	456–470	WLMWRAKGTTGFEAH
	331–345	LVSATAGTTVYGAFD
	551–565	GDKVNFFRMVISNPA

The Binding of g7 and PD Peptide Epitopes of GAD65 to I-A^{g7} and I-A$^{g7.PD}$ MHC Class II Molecules. If allele-specific peptide binding to MHC is found for diabetes-susceptible (g7) and diabetes-resistant (PD) epitopes, it would indicate that epitope selection may play a key role in determining MHC-linked disease susceptibility and resistance. If not, other mechanisms may be involved in the control of MHC-mediated disease susceptibility.

To test the hypothesis of selective binding of T cell epitopes, we measured the relative binding avidity of each g7 and PD epitope to I-A^{g7} and I-A$^{g7.PD}$ molecules by using a competitive peptide–MHC-binding assay. The binding results for the five g7 epitopes showed that some g7 peptides preferentially bound to I-A^{g7} molecules, whereas others did not show such preferential binding (Fig. 3*A*). Three g7 epitopes, p206–220, p286–300, and p401–415, bound preferentially to I-A^{g7} molecules. Peptide 206–220 bound exclusively to I-A^{g7} molecules. Peptide 286–300 and p401–415 bound more strongly to I-A^{g7} than I-A$^{g7.PD}$ molecules. The other g7 peptides (p221–235 and p561–575) did not bind preferentially to either I-A^{g7} or I-A$^{g7.PD}$ molecules. Peptide 221–235 bound strongly to I-A^{g7} and I-A$^{g7.PD}$ molecules, and p561–575 bound weakly to both I-A^{g7} and I-A$^{g7.PD}$ molecules.

Table 4. Relative binding capacity of GAD65 immunogenic epitopes to I-A^{g7} and I-A$^{g7.PD}$ class II molecules

Peptide	Immunogenic epitope of	Binding	
		To I-A^{g7}	To I-A$^{g7.PD}$
206–220	g7	+++	–
221–235	g7	++++	++++
286–300	g7	++++	+
401–415	g7	++++	+++
561–575	g7	+	+
456–470	PD	+	++++
331–345	PD	+	++++
551–565	PD	–	++

++++, 50% inhibition obtained with 0–50 μM inhibitory peptide; +++, 50% inhibition obtained with 51–250 μM inhibitory peptide; ++, 50% inhibition obtained with 251–500 μM inhibitory peptide; +, 50% inhibition obtained with greater than 501 μM inhibitory peptide; –, no inhibition detected.

In contrast to the binding characteristics of the g7 epitopes, all three PD epitopes preferentially bound to I-A$^{g7.PD}$ molecules (Fig. 3*B*). Peptides 456–470 and p331–345 bound much more strongly to I-A$^{g7.PD}$ molecules than to I-A^{g7}. Peptide 551–565 bound exclusively to I-A$^{g7.PD}$ molecules. The IC_{50} for all three PD epitopes binding to M12.C3.g7 are more than 500 μM (data not shown in Fig. 3*B*). Table 4 shows the level of relative binding capacity for g7 and PD epitopes, based on the approximate IC_{50} value for each epitope.

Cytokine Production by T Cells Stimulated with g7 and PD Epitopes. I-A^{g7} and I-A$^{g7.PD}$ molecules identify completely dif-

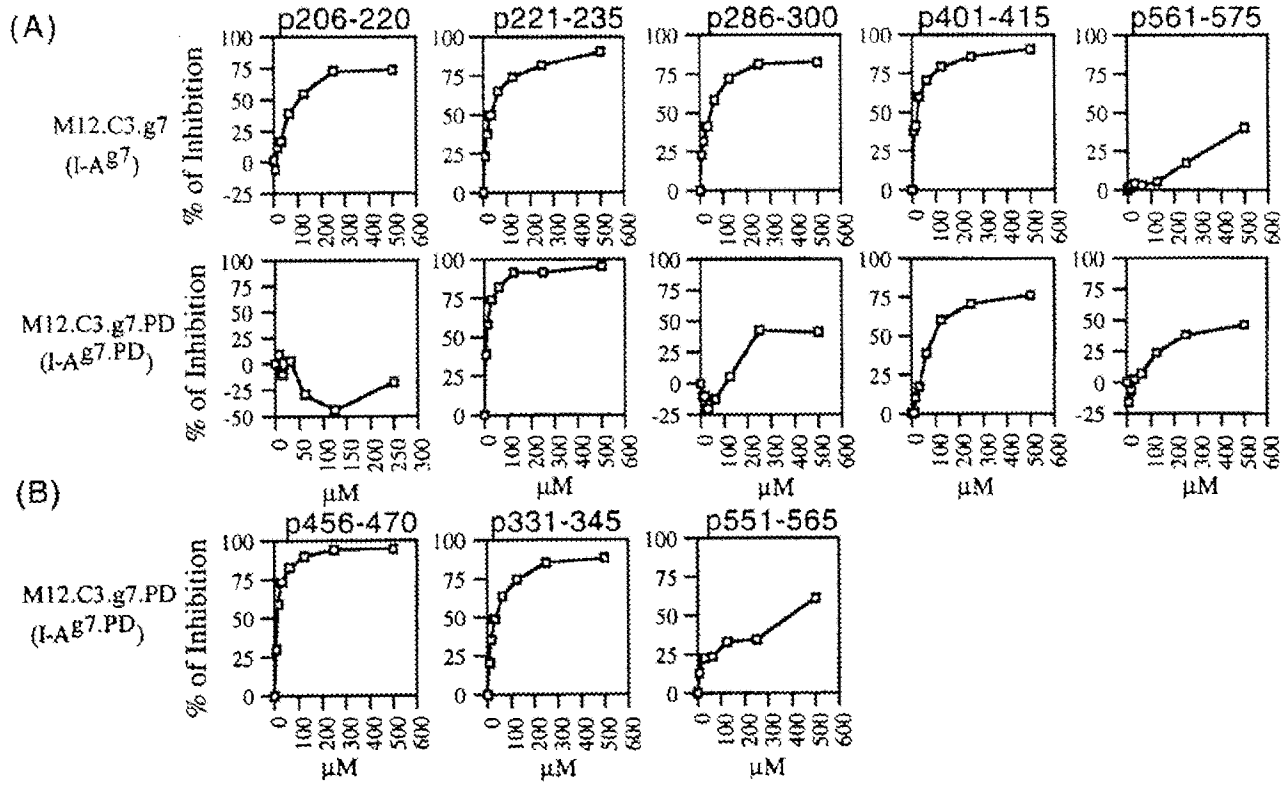

FIG. 3. The binding of g7 (*A*) and PD (*B*) epitopes to I-A^{g7} and I-A$^{g7.PD}$ molecules. M12.C3.g7 and M12.C3.g7.PD cells were used, respectively, as binding targets in this competitive peptide/MHC-binding assay. The inhibitory peptides are shown on the top of each pair of experiments. The *x* axis represents various concentrations (μM) of inhibitory peptides, and the *y* axis represents the percentage of inhibition, which was calculated by the following formula: % of inhibition = $[(R - R_1)/R] \times 100\%$, where R = signal from assays containing only reference peptide, and R_1 = signal from assays containing reference peptide and inhibitory peptide.

Table 5. Cross-immunization experiments

Immunizing peptide	% of peptide-positive hybridomas	% of peptide-specific hybridomas responding to GAD65 protein
456–470 (PD epitope)	448/405 = 89%	0/41 = 0%
331–345 (PD epitope)	458/504 = 91%	0/48 = 0%
206–220 (g7 epitope)	144/168 = 85%	40/48 = 83%

NOD mice were immunized with two PD epitopes (p456–470 or p331–345), 50 μg emulsified in complete Freund's adjuvant (CFA), to generate PD epitope-specific, I-A^{g7} restricted T cell hybridomas. In another group, NOD mice were immunized with a g7 epitope (p206–p220), 50 μg in CFA, to generate p206–220-specific, I-A^{g7}-restricted T cell hybridomas. The percentage of peptide-positive hybridomas represents the ratio of the number of peptide-responding T cell hybridomas to the total number of screened hybridomas. The percentage of peptide-specific hybridomas responding to GAD65 protein represents the ratio of the number of peptide-responding T cell hybridomas responding to GAD65 to the total number of peptide-responding T cell hybridomas that were screened.

ferent sets of immunodominant GAD65 epitopes to T cells, but the functional significance of this difference is not clear. To evaluate the qualitative aspects of GAD65-specific immune responses, NOD wild-type and NOD.PD mice were immunized with 50 μg of GAD65 in IFA, and cytokine production was determined 72 hr after restimulation *in vitro* of T cells from draining lymph nodes with either native antigen, a peptide pool, or individual peptide epitopes.

The cytokine response data (data not shown) demonstrated that large amounts of IFN-γ were secreted by T cells derived from both NOD wild-type and NOD.PD Tg mice when stimulated with GAD65 protein. T cells in NOD mice produced more IFN-γ when stimulated with g7 but not PD peptide pools. Peptide 206–220-specific T cells secreted the highest concentration of IFN-γ among the other g7 epitope-specific T cells derived from NOD mice. T cells in NOD.PD mice produce more IFN-γ when stimulated with PD but not g7 peptide pools. P456–470-specific T cells secreted the highest concentration of IFN-γ among the other PD epitope-specific T cells derived from NOD.PD mice.

Small amounts of IL-4 were secreted by T cells derived from both NOD wild-type and NOD.PD Tg mice when stimulated with GAD65 protein. T cells derived from NOD.PD mice produce slightly more IL-4 than those derived from NOD mice. IL-4 could not be detected when NOD- and NOD.PD-derived T cells were stimulated with peptide pools and individual peptide epitopes.

Cross-Immunization of Wild-Type NOD Mice with PD Epitopes. "Cross-immunization" in this study refers to the injection of PD epitopes into NOD wild-type mice. The purpose of this experiment was to determine whether T cells specific for PD epitopes exist in the periphery of wild-type NOD mice. This procedure might allow us to test the hypothesis of T cell repertoire selection we proposed previously. NOD mice were immunized with two PD epitopes (p456–470 or p331–345) emulsified in complete Freund's adjuvant (CFA) to generate PD epitope-specific, I-A^{g7}-restricted T cell hybridomas. As a positive control, NOD mice were immunized with a g7 epitope (p206–220) in CFA to generate p206–220-specific, I-A^{g7}-restricted T cell hybridomas. The results of cross-immunization experiments are summarized in Table 5. Eighty-nine and 91% of screened T cell hybridomas derived from NOD mice immunized with PD epitopes respond to their priming peptides, p456–470 and p331–345, respectively. Although the PD epitope-specific, I-A^{g7}-restricted T cells exist in the periphery of NOD mice, all of them failed to respond to GAD65 protein at levels as high as 50 μg/ml. In the control group, 85% of screened T cell hybridomas derived from NOD mice primed with p206–220 responded to the given peptide. Unlike PD epitope-specific, I-A^{g7}-restricted T cell hybridomas, the majority (83%) of the g7 epitope-specific, I-A^{g7}-restricted T cell hybridomas responded well to 10 μg/ml GAD65 protein.

DISCUSSION

The experiments reported here demonstrate that I-A^{g7} and I-$A^{g7.PD}$ molecules identify completely different epitopes of GAD65 to T cells. These studies in I-A_g Tg mice clearly demonstrate that a 2-aa difference in the α-helical domain of the β-chains of I-A^{g7} and I-$A^{g7.PD}$ can dramatically affect the specificity of autoreactive T cell responses to the islet cell antigen GAD65. These differences may be central to our understanding of the disease susceptibility mediated by I-A^{g7} and the protective effect mediated by I-$A^{g7.PD}$ alleles.

N- and C-terminal truncation data demonstrate the minimum number of residues required for T cell responses. Although the truncation experiments do not identify the potential sites for T cell and MHC contact with peptides, this information helps us predict a potential peptide-binding motif for I-A^{g7} molecules. I-A^{g7}-binding (19) and HLA-DRB1*0405-binding (20–22) motifs have been identified by acid elution of peptides from MHC molecules. HLA-DRB1*0405 encodes a β-chain lacking an aspartic acid at position 57, similar to the I-A^{g7} allele in NOD mice. In comparing the immunogenic peptides identified in our study with the I-A^{g7} and DRB1*0405 peptide-binding motifs, none of these peptides completely fit the characterized DRB1*0405 and I-A^{g7} motifs. However, it is not unusual for the amino acid sequence of immunogenic epitopes not to correlate with the sequence of predicted motifs. Because the motifs are based on peptide elution studies and thus select for abundant peptides, it is likely that the motifs correspond to peptides that bind with high affinity. It is possible that some autoreactive T cells specific for high-affinity, motif-fitting GAD65 peptides may have been eliminated in the thymus by negative selection. As a result, these peptides may not be detected as immunogenic for peripheral T cells.

The data shown in Fig. 3 and Table 4 demonstrate that peptide binding alone cannot account for the immunodominance of different epitopes of GAD65 identified by I-A^{g7} and I-$A^{g7.PD}$ molecules. Peptide 206–220, the most dominant epitope selected by I-A^{g7} molecules, is not the epitope that has the highest binding affinity among the g7 epitopes. In addition, two g7 epitopes are able to bind well both I-A^{g7} and I-$A^{g7.PD}$ molecules. Even though these two sets of epitopes show some degree of preferential binding to their I-A molecules, only p206–220 (g7 epitope) and p551–565 (PD epitope) show exclusive binding to I-A^{g7} and I-$A^{g7.PD}$ molecules, respectively. These observations imply that other mechanisms are involved in the selection of distinct T cell epitopes by susceptible and resistant MHC alleles.

It has been proposed that the development of insulin-dependent diabetes is controlled by the Th1 vs. Th2 phenotype of autoreactive Th cells: Th1 cells would promote diabetes, whereas Th2 cells would protect from disease (23, 24). However, cytokine experiments reveal that there are no qualitative differences in the cytokine profiles of T cells derived from NOD and NOD.PD mice after stimulation with GAD65. T cells from both strains produce Th1-like cytokines. It is surprising that a large amount of Th1 cytokine (IFN-γ) was produced by GAD65-specific T cells in diabetes-resistant NOD.PD mice. One explanation may be that although GAD65-specific T cells secrete inflammatory Th1 cytokines in NOD.PD mice, other islet antigen-specific T cells may secrete Th2-like cytokines that delay or block the process of type I diabetes. The age of the mice is also critical for the disease process. T cells of the same antigen specificity may have different cytokine profiles at different ages. Furthermore, immunogenic epitopes from autoantigens have been shown to differ in mice of different ages because of epitope spreading (13). It is also possible that distinct T cell epitopes of islet cell antigens may elicit different effector functions.

Cross-immunization experiments with the PD epitope p456–470 demonstrated that this peptide is also immunogenic in wild-type NOD mice if the peptide epitope is used for immunization and T cell stimulation. However, I-A^{g7}-restricted T cell

hybridomas specific for p456–470 (which were not identified in NOD wild-type mice after immunization with native protein) responded only to synthetic peptide, not the protein antigen. In identical studies of the second PD epitope, p331–345, similar findings were observed. Why do PD peptide-specific, I-A^{g7}-restricted T cell hybridomas respond only to GAD65 peptide, but not whole protein? Three possibilities may explain this phenomenon. First, the PD epitopes may be functionally recessive (cryptic) in NOD mice. In other words, these two PD epitopes may not be processed and presented efficiently on the surface of I-A^{g7}-expressing APC in NOD wild-type mice. Thus, antigen-specific T cells never have the opportunity to meet the processed peptide antigens. It is evident that the hierarchy of display of peptide determinants on a protein antigen can differ greatly in different alleles of MHC molecules, as well as in the same MHC molecule on APC in different locations (25, 26).

Second, differences may exist in the frequency of peripheral TCR in NOD wild-type and NOD.PD Tg mice that are specific for the naturally processed peptide/MHC complex (derived from protein antigen), the synthetic peptide/MHC complex, or both. Viner *et al.* (27) showed that the interaction of free peptides with class II MHC molecules can generate complexes that are antigenically dissimilar to those resulting from intracellular processing of intact antigens. It is possible that the T cells responding to synthetic peptide antigens are different from T cells that are able to recognize naturally processed epitopes. This phenomenon has been described as type A (response to peptide and protein antigen) and type B (exclusively to peptide) T cells by Unanue and his colleagues (28). The third possibility is that these PD epitope-specific, I-A^{g7}-restricted T cells have a very low binding affinity to PD epitopes on I-A^{g7} molecules. Therefore, the T cells responding to PD epitopes in NOD mice can be detected only after administration of a large amount of peptides in adjuvant or aqueous form (data not shown).

Based on the results of this study, it is possible that selective binding of T cell peptide epitopes, the ability to process/present antigen, and selection of a distinct T cell repertoire may all be involved in the control of MHC-linked susceptibility. Susceptibility alleles (I-A^{g7}) predispose to type 1 diabetes by allowing binding and presentation of pathogenic self-peptides as well as allowing the development of an autoreactive T cell repertoire in the periphery. In contrast, the lack of pathogenic T cell responses in resistant strains (individuals) is due to the absence or suppression of one or more of these three capabilities. Furthermore, the control mechanisms involved in the selection of autoantigen T cell epitopes are complex and appear to differ for each peptide epitope. For p206–220, peptide binding plays an important role in recognition of this epitope by T cells in diabetes-susceptible mice. On the other hand, p221–235 binds equally well to I-A^{g7} and I-$A^{g7.PD}$ molecules. Therefore, other mechanisms also can play critical roles in MHC control of disease susceptibility. These include on/off rate of the peptide/MHC complex, the half-life of this complex, and the effect of H-2M molecules on antigen presentation by susceptible and resistant alleles. All of these factors can have major effects on peptide/MHC complex surface expression and on Th1/Th2 differentiation.

The present experiments cannot resolve these three possibilities. To further analyze this problem, it will be necessary to measure on/off rates of individual peptide epitopes for each MHC allele; the effect of H-2M molecules on antigen presentation; and the time course of the peptide-specific T cell response and its functional characteristics, by using tetramers of I-A^{g7}- and I-$A^{g7.PD}$-specific peptide complexes, to trace each antigen-specific T cell. Generation of TCR Tg mice for the three g7- and two PD-dominant epitopes will permit further characterization of the response to these five epitopes. The knowledge from these studies will help us understand how I-A alleles predispose to or prevent the development of type 1 diabetes.

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Correction

IMMUNOLOGY. In the article "The role of MHC class II molecules in susceptibility to type I diabetes: Identification of peptide epitopes and characterization of the T cell repertoire" by Cheng-Chi Chao, Huey-Kang Sytwu, Emily Lichuan Chen, Jon Toma, and Hugh O. McDevitt, which appeared in number 16 of *Proc. Natl. Acad. Sci. USA* (**96**, 9299–9304), the following corrections are noted.

Four amino acid sequences in Table 3 were incorrect. The corrections are as follows:

(i) For peptide 206–220, the fourth amino acid of this sequence is I, not L.

(ii) For peptide 221–235, the sixth amino acid of this sequence is E, not F.

(iii) For peptide 286–300, the seventh amino acid of this sequence is L, not I.

(iv) For peptide 401–415, the third amino acid of this sequence is Q, not O.

EXHIBIT D

TCR Agonist and Antagonist Exert In Vivo Cross-Regulation When Presented on Igs[1]

Kevin L. Legge, Booki Min, Aimee E. Cestra, Christopher D. Pack, and Habib Zaghouani[2]

Ig-PLP1 and Ig-PLP-LR are chimeric Igs expressing proteolipid protein (PLP)-derived T cell agonist (PLP1) and antagonist (PLP-LR) peptides, respectively. Both chimeras, like free PLP1 and PLP-LR peptides, induce in vivo-specific T cell responses. However, the responses induced by Ig-PLP1 and Ig-PLP-LR were cross-reactive with both PLP1 and PLP-LR peptides, while those induced by free peptides were not. Surprisingly, despite the cross-reactivity of the responses, when Ig-PLP1 and Ig-PLP-LR were administered together into mice, a dose-dependent down-regulation of both T cell responses and a reduction of IL-2 production to background levels was observed. In contrast, when T cells induced by either Ig chimera were stimulated in vitro with mixtures of free PLP1 and PLP-LR peptides, there was no down-regulation of proliferation or decrease in IL-2 production. These data indicate that Ig-PLP1 and Ig-PLP-LR exert adverse reactions on one another at the level of naive T cells, resulting in an opposite antagonism. However, naive T cells experiencing either chimera develop into cross-reactive cells, acquire resistance to TCR triggering by closely related but different peptides, and support responsiveness. *The Journal of Immunology*, **1998, 161: 106–111.**

Altered peptides mutated at the TCR contact residue(s) bind to MHC molecules equally as well as the immunogenic peptides, yield functional ligands that engage the TCR, and support overall T cell recognition (1–7). These ligands can function as T cell antagonists (2, 3, 5, 7–10), partial agonists (9–11), or super agonists (12). While partial and super agonism could result from a readjustment of the signaling cascade (reviewed in Ref. 13), antagonism may be the consequence of TCR spoiling, a phenomenon referring to TCR occupancy that triggers no signal or an unproductive one at best (14, 15). Proteolipid protein (PLP)[3]-derived T cell agonist (PLP1) peptide, encompassing amino acid (aa) residues 139–151 of PLP, functions as an agonist and induces encephalitogenic T cells in SJL/J ($H\text{-}2^s$) mice (16, 17). Replacing 144W and 147H with 144L and 147R, respectively, within PLP1 generates an antagonist peptide, PLP-LR, that interferes with TCR triggering by PLP1 and inhibits T cell activation (7). Because Igs internalize into APCs via FcRs, access the endocytic pathway for presentation, and reach newly synthesized MHC molecules (18–20), both PLP1 and PLP-LR were expressed on Igs. The resulting Ig-PLP1 and Ig-PLP-LR provided a system to assay for antagonism at the endocytic level as might be required for the effective amelioration of T cell-mediated autoimmune diseases (21). Ig-PLP1 was presented via the endocytic pathway and was a potent activator of T cell hybridomas specific for PLP1 peptide (21). Similarly, Ig-PLP-LR was efficient in peptide loading onto MHC class II molecules and was shown to function as an antagonist. Indeed, Ig-PLP-LR efficiently inactivated PLP1-specific T cell hybridomas, regardless of whether they were stimulated with free PLP1 peptide or with Ig-PLP1 chimera (21). In vivo, the coadministration of 50 μg of Ig-PLP1 with 150 μg of Ig-PLP-LR into SJL/J mice resulted in a reduction to a background (BG) level of response to PLP1 peptide but yielded significant proliferation to PLP-LR peptide (21). These observations suggested that Ig-PLP-LR was either spoiling TCRs on naive T cells or down-regulating the PLP1-specific T cells induced by Ig-PLP1. In an effort to understand the underlying mechanism of in vivo T cell antagonism, proliferative and cytokine responses were measured in mice that had been immunized with individual Ig-PLP chimeras or varying mixtures of Ig-PLP1 and Ig-PLP-LR. We discovered that Ig-PLP-LR given alone to mice induced T cells that, like those induced by Ig-PLP1, cross-reacted with both PLP1 and PLP-LR peptides. Surprisingly, however, the chimeras displayed a dose-dependent antagonism on one another when coadministered, despite the cross-reactivity of the responses; this antagonism resulted in a down-regulation of both T cell responses. Finally, Ag-specific T cells induced either by Ig-PLP1 or by Ig-PLP-LR were refractory to down-regulation by peptide mixtures and proliferated significantly when they were in vitro-stimulated simultaneously with both PLP1 and PLP-LR. These findings indicate that both agonist and antagonist peptides exert adverse reactions on one another and reveal an opposite antagonism and a stringent control of TCR triggering at the level of naive T cells.

Materials and Methods

Mice

We purchased 5- to 8-wk-old SJL/J mice ($H\text{-}2^s$) from Harlan-Sprague-Dawley (Frederick, MD). The mice were housed in our animal facility for the duration of these experiments.

Antigens

Peptides. All of the peptides used in this study were purchased from Research Genetics (Huntsville, AL) and purified by HPLC to >90% purity. PLP1 peptide (HSLGKWLGHPDKF) corresponds to aa residues 139–151 of PLP. PLP1 is a T cell peptide that is presented by $I\text{-}A^s$ class II molecules and is encephalitogenic in $H\text{-}2^s$ mice (16, 17). PLP-LR peptide (HSL

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[2] Address correspondence and reprint requests to Dr. Habib Zaghouani, University of Tennessee, Department of Microbiology, M409 Walters Life Sciences Building, Knoxville, TN 37996. E-mail address: hzagh@utk.edu

[3] Abbreviations used in this paper: PLP, proteolipid protein; PPD, purified protein derivative; aa, amino acid; BG, background.

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GKLLGRPDKF) is a mutant form of PLP1, in which the major TCR-contacting residues, Trp144 and His147, were replaced with Leu and Arg, respectively (7). PLP-LR binds to I-A^s equally as well as PLP1 and has been defined as a TCR antagonist peptide (7). PLP2 peptide (NTWTTC QSIAFPSK) corresponds to aa residues 178–191 of PLP and is also presented by I-A^s class II molecules (22).

Ig-PLP chimeras. Nucleotide sequences encoding PLP1, PLP-LR, and PLP2 peptides were inserted in place of the D segment within the complementarity determining region 3 of the V_H gene of the 91A3 anti-arsonate Ab using a previously described PCR mutagenesis procedure (23). The 91A3 V_H-PLP chimeric genes were then ligated to the exons encoding a BALB/c γ2b constant region (Cγ2b) to form complete heavy chains (21). The 91A3 V_H-PLP-Cγ2b genes were cotransfected with the parental 91A3 light chain gene into the non-Ig-secreting myeloma B cell line SP2/0 to express complete Ig molecules. The resulting Ig chimeras were designated Ig-PLP1, Ig-PLP-LR, and Ig-PLP2, respectively. Transfectoma cells producing 2 to 4 μg/ml of Ig-PLP chimera were grown to large scale in 2-L roller bottles, and the chimeras were purified from culture supernatant by affinity chromatography on columns made of rat anti-mouse κ chain coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) as described previously (23). Each Ig chimera was purified using separate columns to avoid cross contamination.

Immunization of mice with Ig chimeras

Mice (five per group) were immunized s.c. in the foot pads and at the base of the limbs and tail with Ig-PLP chimera emulsified in a 200 μl mixture of PBS/CFA (1:1 v/v). The mice were sacrificed after 10 days by cervical dislocation, the spleens and lymph nodes (axillary, lateral axillary, and popliteal) were removed, single-cell suspensions were prepared, and the T cell proliferative response and cytokine production were assessed as described below.

Assays for spleen and lymph node proliferative responses

Lymph node and spleen cells were incubated in 96-well flat-bottom plates at 4 and 10 × 10^5 cells/100 μl/well, respectively, with 100 μl of stimulator for 3 days. Subsequently, 1 μCi of [^{3}H]thymidine was added per well, and the culture was continued for an additional 14.5 h. The cells were subsequently harvested on glass fiber filters, and incorporated [^{3}H]thymidine was counted using the trace 96 program and an Inotech beta counter (Wohlen, Switzerland). Unless indicated otherwise, the stimulators were used at the following defined optimal concentrations: PLP1, PLP-LR, and PLP2 peptides at 15 μg/ml and purified protein derivative (PPD) of *Mycobacterium tuberculosis* at 5 μg/ml.

ELISA screening for spleen cytokine production

Spleen cells were incubated in 96-well round-bottom plates at 10 × 10^5 cells/100 μl/well with 100 μl of stimulator, as described above, for 24 h. Cytokine production was measured by ELISA according to the manufacturer's instructions (PharMingen, San Diego, CA) using 100 μl of culture supernatant. The capture Abs used were rat anti-mouse IL-2 (JES6-1A12), rat anti-mouse IL-4 (11B11), rat anti-mouse IFN-γ (R4–6A2), and rat anti-mouse IL-10 (JES5-2A5). The biotinylated anti-cytokine Abs used were rat anti-mouse IL-2 (JES6-5H4), rat anti-mouse IL-4 (BVD6-24G2), rat anti-mouse IFN-γ (XMG1.2), and rat anti-mouse IL-10 (JES5-16E3). The OD_{405} was measured on a SpectraMAX 340 counter (Molecular Devices, Menlo Park, CA) using SoftMAX PRO version 1.2.0 software. Graded amounts of mouse rIL-2, rIL-4, rIFN-γ, and rIL-10 were included in all experiments to construct standard curves. The concentration of cytokines in culture supernatants was estimated by extrapolation from the linear portion of the standard curve.

Results

We have demonstrated previously that Ig-PLP1 is presented to PLP1-specific T cell hybridomas (21). Evidence for the presentation of Ig-PLP-LR arose from the observation that Ig-PLP-LR inhibited the activation of PLP1-specific T cell hybridomas during stimulation with free PLP1 peptide or Ig-PLP1. (21). Injecting Ig-PLP1 into SJL/J mice induced prominent PLP1-specific lymph node and splenic T cell proliferative responses (21). The coinjection of Ig-PLP-LR with Ig-PLP1 markedly reduced the response to PLP1 (21). In these mice, however, we observed significant proliferative lymph node responses to PLP-LR peptide. We then formulated the hypothesis that Ig-PLP-LR was either spoiling TCRs on PLP1-specific naive T cells or inducing T cells that have a down-regulatory effect on Ig-PLP1-induced T cells. To investigate the in vivo down-regulatory effect of Ig-PLP-LR on Ig-PLP1, we proceeded to determine whether Ig-PLP-LR could induce a specific T cell response, and, if so, how this response would compare with the response to Ig-PLP1. Furthermore, we sought to determine whether the T cell down-regulation was dependent upon the dose of Ig-PLP-LR. Accordingly, mice were immunized with Ig-PLP1, Ig-PLP-LR, PLP1, or PLP-LR, and their lymph node proliferative responses to free PLP1 and PLP-LR peptides were measured. The data illustrated in Figure 1 indicate that Ig-PLP1, like PLP1 peptide, induced a specific T cell response to PLP1 peptide. Similarly, Ig-PLP-LR, like PLP-LR peptide, induced a specific T cell response to PLP-LR peptide. Neither the Ig chimera nor the free peptides induced T cells that significantly reacted with the negative control PLP2, a peptide that is also presented by I-A^s class II molecules. Surprisingly, however, the response induced by Ig-PLP1 cross-reacted with PLP-LR peptide, while the response induced by Ig-PLP-LR cross-reacted with PLP1. The responses induced with free PLP1 or free PLP-LR were not cross-reactive under these experimental conditions (Fig. 1). Stimulation of cells from PLP-LR-immunized mice with a higher concentration of PLP1 peptide resulted in low but significant proliferation (24). These observations prompted us to investigate whether Ig-PLP-LR acts on Ig-PLP1 in a dose dependent-fashion, and whether it is subjected to counterregulation by Ig-PLP1. As can be seen in Figure 2, lymph node T cells from a new group of mice that were immunized with Ig-PLP1 proliferated equally well to free PLP1 and PLP-LR peptides. Splenic T cells from these mice failed to respond to free PLP-LR peptide stimulation (Fig. 3). However, when an additional group of mice was immunized with Ig-PLP-LR, both lymph node and splenic cells proliferated to PLP1 as well as to PLP-LR peptide (Figs. 2 and 3). Ig-W, a wild-type molecule not carrying any PLP peptide, failed to induce a T cell response in either lymphoid tissue (Figs. 2 and 3). When mice were immunized with a mixture containing equal amounts of Ig-PLP1 and Ig-PLP-LR, the T cell responses were greatly decreased in comparison with immunization with either chimera alone (Figs. 2 and

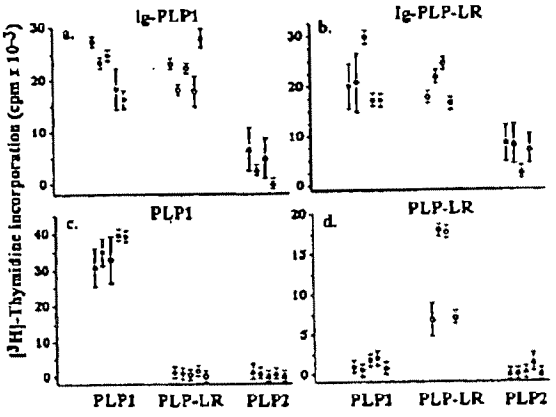

FIGURE 1. Lymph node proliferative responses to immunization with Ig-PLP chimeras. Mice were injected with 50 μg of Ig-PLP1 (*a*), 50 μg of Ig-PLP-LR (*b*), 100 μg of PLP1 (*c*), or 100 μg of PLP-LR (*d*) in CFA; After 10 days, the lymph node cells were in vitro-stimulated with 15 μg/ml of free PLP1 (●), PLP-LR (○), or PLP2 (△). The resulting proliferation was measured by [^{3}H]thymidine incorporation. Cells incubated without stimulator were used as BG. Each mouse was individually tested in triplicate wells for each stimulator, and the indicated cpms represent the mean ± SD after the deduction of BG cpms.

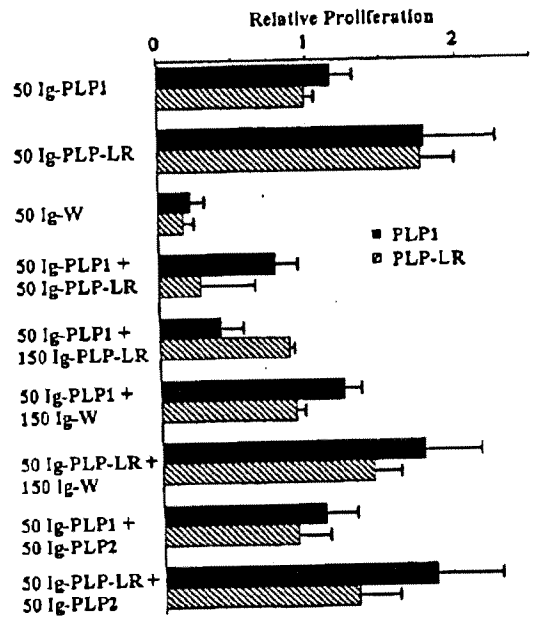

FIGURE 2. Lymph node T cell proliferative response to coimmunization with Ig-PLP1 and Ig-PLP-LR. Mice (five per group) were injected with a single Ig chimera or with mixtures of Ig chimeras; After 10 days, the lymph node cells were in vitro-stimulated with free PLP1 (filled bars) or PLP-LR (hatched bars) and assayed for proliferation by [^{3}H]thymidine incorporation. The number preceding the Ig chimera label indicates the amount of micrograms that were injected per mouse. The stimulators were PPD at 5 μg/ml or PLP1, PLP-LR, and PLP2 at 15 μg/ml. Cells incubated without stimulator were used as BG. The mice were tested individually, and triplicate wells were assayed for each stimulator. To standardize the results and eliminate intrinsic individual variability, we expressed the results as a relative proliferation that was estimated as follows: (mean test peptide cpm − mean BG cpm)/(mean PPD cpm − mean BG cpm). The indicated relative proliferation represents the mean ± SD of five mice tested individually. The mean cpms ± SD that were obtained with PPD stimulation for the different groups of mice were as follows: 50 μg of Ig-PLP1: 16,413 ± 1,330; 50 of μg Ig-PLP-LR: 11,224 ± 3,481; 50 μg of Ig-W: 11,513 ± 1,572; 50 μg of Ig-PLP1 plus 50 μg of Ig-PLP-LR: 16,817 ± 2,869; 50 μg of Ig-PLP1 plus 150 μg of Ig-PLP-LR: 16,156 ± 2,006; 50 μg of Ig-PLP1 plus 150 μg of Ig-W: 11,699 ± 1,142; 50 μg of Ig-PLP-LR plus 150 μg of Ig-W: 13,435 ± 1,650; 50 μg of Ig-PLP1 plus 50 μg of Ig-PLP2: 10,056 ± 1,407; and 50 μg of Ig-PLP-LR plus 50 μg of Ig-PLP2: 10,877 ± 563. The bars indicate the standardized proliferation to free PLP1 and PLP-LR peptides. The proliferation to PLP2 peptide was at BG levels except where Ig-PLP2 was used in the immunization mixture.

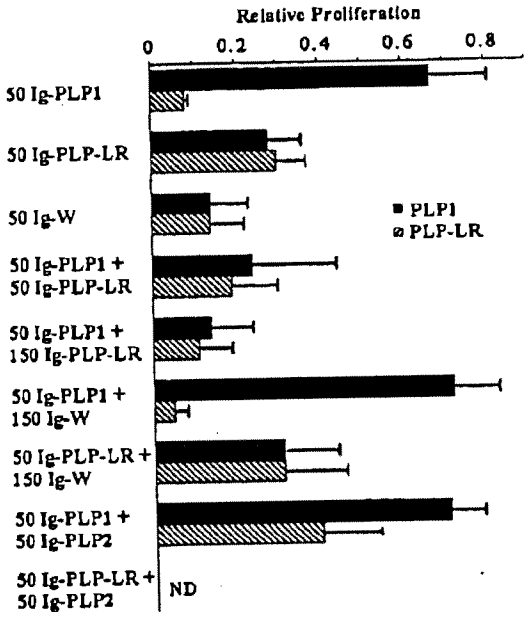

FIGURE 3. Splenic proliferative T cell responses of mice coimmunized with Ig-PLP1 and Ig-PLP-LR. Spleen cells from the mice described in Figure 2 were stimulated with free PLP1 (filled bars) or PLP-LR (hatched bars) in triplicate wells, and proliferation was measured as described above. The results were standardized as described above using PPD cpms obtained with lymph node T cells, because the proliferation of spleen cells upon stimulation with PPD was minimal. The indicated relative proliferation represents the mean ± SD of five individually tested mice.

3). Furthermore, the PLP1-specific lymph node responses were lower than responses obtained in mice immunized with Ig-PLP1 alone and represented only 50% of the PLP1-specific lymph node response of mice immunized with Ig-PLP-LR. Surprisingly, the PLP-LR response was at BG levels (Fig. 2). Accordingly, although the responses to the Ig chimeras share cross-reactivity between PLP1 and PLP-LR peptides, immunization with mixtures of Ig chimeras yielded down-regulation rather than additive responses. In fact, the data argue for an opposite down-regulation among Ig-PLP1 and Ig-PLP-LR. This down-regulation appeared to be dose-dependent, because mice that were injected with a mixture of 50 μg of Ig-PLP1 and 150 μg of Ig-PLP-LR failed to respond to PLP1 and mounted responses to PLP-LR that were reduced to the levels observed with mice injected with Ig-PLP1 alone (Fig. 2). Previously, we had concluded that this PLP-LR response was normal (21), but in comparison with the responses of animals injected with Ig-PLP-LR alone, there is a 50% reduction indicating the down-regulatory effect of Ig-PLP1 on a high dose of Ig-PLP-LR (Fig. 2).

Neither the PLP1 nor the PLP-LR response was affected when Ig-PLP1 or Ig-PLP-LR was coinjected with the wild-type molecule Ig-W (Fig. 2). Similarly, when Ig-PLP1 and Ig-PLP-LR were separately coinjected into animals with Ig-PLP2, a chimera presented by I-A^s class II molecules, the response to either peptide was unaffected (Fig. 2).

In the spleen, like in the lymph nodes, the proliferative responses were not additive (Fig. 3). Rather, an opposite down-regulatory effect between Ig-PLP1 and Ig-PLP-LR was seen. Although a coinjection of Ig-W with either Ig-PLP1 or Ig-PLP-LR did not affect either response, a coinjection of Ig-PLP2 with Ig-PLP1 increased reactivity to PLP-LR among the T cells induced by Ig-PLP1. Whether or not a bystander effect (24, 25) emanating from PLP2-induced T cells helped Ig-PLP1-induced PLP-LR-reactive T cells to migrate to the spleen remains to be investigated.

To further investigate the opposing down-regulation among Ig-PLP1 and Ig-PLP-LR, splenic Ag-induced cytokine responses were measured in animals immunized with either a single Ig chimera or a combination of both. Upon in vitro stimulation with PLP1 peptide, T cells from Ig-PLP1-immunized mice produced IL-2, IFN-γ, and small amounts of IL-4 (Fig. 4). However, stimulating the same cells with PLP-LR yielded minimal IL-2 and undetectable IFN-γ or IL-4 (Fig. 4). Spleen cells from Ig-PLP-LR-immunized mice generated IL-2 but no IFN-γ or IL-4 upon

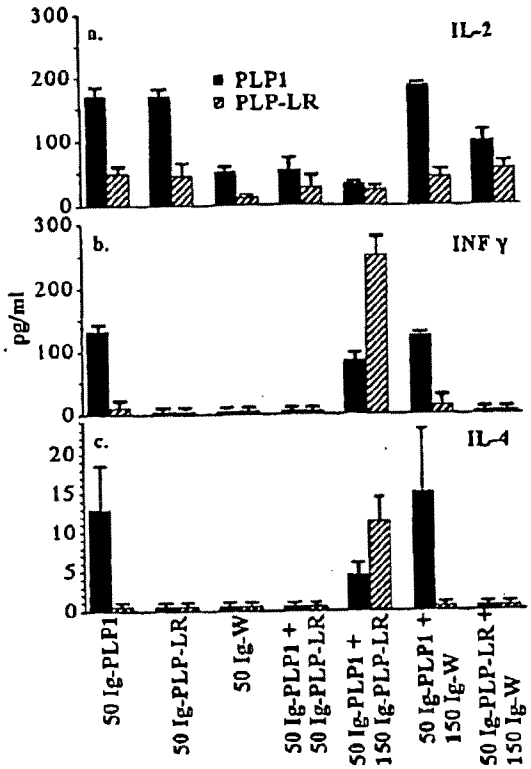

FIGURE 4. IL-2 production by the splenic cells of mice coimmunized with Ig-PLP1 and Ig-PLP-LR. Spleen cells (1×10^6 per well) from the mice described in Figure 2, which were immunized with single Ig chimeras or mixtures of Ig chimeras, were stimulated with PLP1 (filled bars) or PLP-LR (hatched bars) for 24 h; the production of IL-2 (*a*), IFN-γ (*b*), and IL-4 (*c*) was measured by ELISA as described in *Materials and Methods*. Lymph node cells were also assayed for cytokine production by ELISA, but the results were low and were not reproducible (most likely related to highly proliferative T cells reabsorbing the cytokines produced). Triplicate wells were used for each peptide stimulation. Cells incubated without stimulator peptide were used as BG. The indicated amounts represent the mean ± SD of five individually tested mice. The production of IL-10 was also measured, but the results were at BG levels (data not shown).

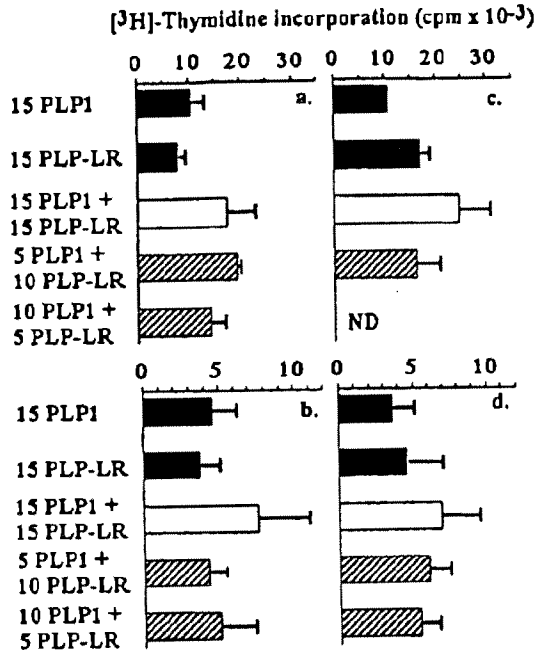

FIGURE 5. Proliferation of Ag-experienced T cells upon stimulation in vitro with mixtures of PLP1 and PLP-LR peptides. Mice (four per group) were immunized with 50 μg of Ig-PLP1 (*a* and *b*) or 50 μg of Ig-PLP-LR (*c* and *d*) in CFA; after 10 days the lymph node (*a* and *c*) and spleen (*b* and *d*) cells were subsequently stimulated with single or mixtures of free peptides, as indicated to the left of each bar, and assayed for [^{3}H]thymidine incorporation as described above. The number preceding the peptide label indicates the amount of micrograms per milliliter that were used for in vitro stimulation. The specific proliferation was estimated by deducting the mean BG (obtained by incubating cells without stimulator) cpm from the test sample cpm. The indicated cpms represent the mean ± SD of four individually tested mice.

stimulation with PLP1 peptide. Moreover, PLP-LR peptide stimulation produced only a minimal IL-2 response. In mice immunized with equal amounts of Ig-PLP1 and Ig-PLP-LR, all cytokine production was reduced to minimal or BG levels upon stimulation with either peptide. Coinjecting Ig-W with either chimera had no measurable effect on the cytokine production pattern (Fig. 4). Significant amounts of IL-4 and IFN-γ were evident upon stimulation with PLP-LR peptide when the animals were given a 3:1 ratio of Ig-PLP-LR to Ig-PLP1, although the splenic proliferative responses and IL-2 production were at BG levels (Fig. 4). Consequently, the excess of Ig-PLP-LR may lead to a mixed but PLP-LR-dominant TCR triggering that induces cells which are able to produce cytokine but which exhibit no proliferative response. Incomplete or mixed signaling, which is a form of interference with signal one, was shown to have a more pronounced down-regulatory effect on proliferation than on cytokine production (1). None of the immunization regimens illustrated in Figure 4 induced detectable levels of IL-10 (data not shown).

To investigate whether Ig-PLP1 and Ig-PLP-LR could display similar adverse reactions on each other at the level of Ag-experienced, cross-reactive T cells, we immunized mice with Ig-PLP1 or Ig-PLP-LR alone and assessed the proliferative T cell responses and IL-2 production upon in vitro stimulation with varying mixtures of free PLP1 and PLP-LR peptides. As can be seen in Figure 5, both lymph node and spleen cells from mice immunized with Ig-PLP1 or Ig-PLP-LR proliferated equally as well to stimulation with a single peptide as to a mixture of PLP1 and PLP-LR. The proliferative response to the mixture, in most cases, was even higher than the response to a single peptide stimulation (Fig. 5). Similarly, IL-2 production was not decreased when spleen cells were stimulated with varying mixtures of PLP1 and PLP-LR (Fig. 6). On the contrary, IL-2 production was higher in most cases of stimulation with peptide mixture than stimulation with a single peptide. Although both proliferation and IL-2 production were often higher when the stimulation was conducted with the peptide mixture, additive responses were not observed.

Discussion

The data reported here show that both Ig-PLP1 and Ig-PLP-LR are immunogenic and induce peptide-specific T cell responses when injected into compatible mice in CFA. Surprisingly, however, both Ig-PLP1 and Ig-PLP-LR induced lymph node T cell responses that

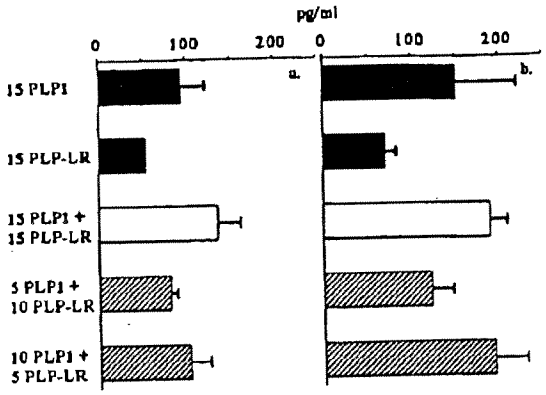

FIGURE 6. IL-2 production by Ag-experienced T cells upon in vitro stimulation with PLP1/PLP-LR peptide mixtures. Spleen cells from Ig-PLP1 (*a*) and Ig-PLP-LR (*b*) immunized mice were in vitro-stimulated with the indicated peptides (illustrated to the left of each bar) and tested for IL-2 production by ELISA as described in Figure 4. The spleen cells used in these experiments were obtained from the mice described in Figure 5. The number preceding the name of the peptide represents the amount of micrograms per milliliter used for stimulation. The indicated pg/ml IL-2 values represent the mean ± SD of four individually tested mice.

were cross-reactive (Fig. 1). Indeed, T cells from mice immunized with either Ig-PLP1 or Ig-PLP-LR reacted with both PLP1 and PLP-LR (Fig. 1). This cross-reactivity was specific to PLP1 and PLP-LR peptide and was not observed when the cells were stimulated with PLP2, a peptide also presented by I-A^s class II molecules like PLP1 and PLP-LR. The splenic T cell responses of mice immunized with Ig-PLP-LR were also cross reactive with both peptides, while the responses induced with Ig-PLP1 showed no cross-reactivity with PLP-LR. The mechanism underlying the loss of cross-reactivity among Ig-PLP1-induced T cells subsequent to migration to the spleen is unknown.

Efficient peptide loading onto MHC class II molecules by the Ig chimeras could generate significant amounts of MHC/peptide complexes (19). In addition, the binding and internalization of Igs into APCs via FcRs may up-regulate the expression of costimulatory molecules. Under these circumstances, an immunization with Ig-peptide chimeras would be able to prime a larger T cell repertoire, including low affinity T cells.

When Ig-PLP1 and Ig-PLP-LR were administered together into mice, the lymph node as well as the splenic proliferative T cell responses were markedly reduced (Figs. 2 and 3). In addition, IL-2 production was reduced to BG levels (Fig. 4). These data indicated that Ig-PLP1 and Ig-PLP-LR exerted adverse reactions on one another, leading to the down-regulation of both T cell responses. Competition for internalization into APCs via FcRs cannot account for the opposing effects between Ig-PLP1 and Ig-PLP-LR, because coinjecting either chimera with Ig-W, the parental Ig encompassing an identical Fc region (the site that mediates binding to FcRs) as Ig-PLP1 and Ig-PLP-LR had no down-regulatory effect on either T cell response (Figs. 2 and 3). Similarly, since PLP2 peptide, like PLP1 and PLP-LR, is presented by I-A^s class II molecules (22), and because Ig-PLP2 had no effect on the responses induced by Ig-PLP1 or Ig-PLP-LR, the opposite down-regulation between Ig-PLP1 and Ig-PLP-LR would appear to be Ag-specific and would most likely not involve competition for binding to I-A^s class II molecules (Figs. 2 and 3).

The explanation we wish to put forth for this opposite down-regulation between Ig-PLP1 and Ig-PLP-LR is that clonal expansion requires an optimal serial triggering with an homogeneous peptide (i.e., all or most of the receptors on a single naive T cell must engage one type of peptide to expand). The simultaneous stimulation of naive T cells with peptides encompassing subtle differences at the TCR contact residues, which may be occurring during immunizations involving mixtures of Ig-PLP1 and Ig-PLP-LR, fails to cause T cell expansion and in vitro proliferation.

It was previously demonstrated that PLP1-specific T cell hybridomas generated from T cell clones obtained by immunization with free PLP1 peptide were not cross-reactive with PLP-LR (7). Rather, the hybridomas were antagonized by PLP-LR (7). Similarly, an interaction of these T cell hybridomas with Ig-PLP-LR led to their inactivation and to an inhibition of cytokine production (21). The T cells induced by either Ig-PLP1 or Ig-PLP-LR were, as demonstrated in Figure 1, cross-reactive with both peptides, and these T cells proliferate and produce cytokines in response to stimulation with either peptide. Because of this cross-reactivity, these T cells proliferated and produced cytokines when they were stimulated with a mixture of PLP1 and PLP-LR in vitro (Figs. 5 and 6). All of the cells in a single Ig-peptide chimera immunization regimen are primed by one peptide, and in vitro stimulation of these cells with a mixture of peptides neither inhibited nor led to additive responses. These results suggest that the response induced by immunization with a single Ig-peptide chimera comprises T cells expressing cross-reactive TCR rather than distinct populations specific for individual peptides. Consequently, these Ag-experienced, cross-reactive T cells, unlike naive T cells, are resistant to mixed TCR triggering by PLP1 and PLP-LR. Monoclonal T cells, whether clones or hybridomas, that are generated by repeated in vitro peptide stimulation are likely to be more sensitive to antagonism because of their higher affinity to the selecting peptide. PLP1-specific T cell hybridomas and lines were in fact antagonized by Ig-PLP-LR and PLP-LR peptide (7, 21).

Overall, naive T cells, although potentially cross-reactive with both agonist and antagonist peptides, resist mixed TCR triggering during the first Ag exposure and appear to support an opposite down-regulation among peptides with subtle differences at the TCR contact residues. A cell-signaling analysis has demonstrated that TCR occupancy without a sustained calcium signal could form the basis for TCR spoiling and antagonist interference with TCR triggering by an agonist (14, 15). Some altered peptides, however, may trigger signals that could support distinct or partial activation patterns (reviewed in Ref. 13). In our case of opposite antagonism, one may speculate that both PLP1 and PLP-LR, when presented by Igs, trigger productive signals that lead to T cell activation and expansion, as evidenced by the induction of specific T cell responses. These signals could be closely related and support cross-reactivity at the level of Ag-experienced T cells. However, naive T cells undergoing mixed signaling are discriminatory and may not tolerate these closely related signals emanating from peptides with such subtle aa differences. Whether this observation implies that the signaling machinery, exerting a stringent control of TCR triggering at the level of naive T cells, evolves with some flexibility to support cross-reactivity by Ag-experienced T cells remains to be investigated. However, it has been previously suggested that neuroantigen-specific naive T cells have more stringent activation requirements than T cells that have encountered the Ag (26). In addition, it was recently demonstrated that T cells can be activated by peptides that are unrelated in sequence to their selecting peptide (27). Finally, we would like to emphasize that the delivery of peptides on Igs for active immunization could result in responses that are qualitatively different from those induced by free peptides (Fig. 1). The binding of Igs to FcRs on APCs recruited to the site of injection by CFA could trigger specific factors that could influence

T cell-APC interactions, possibly resulting in the preferential expansion of cross-reactive T cells. In the absence of adjuvant, the enhanced presentation of antagonist peptides by Igs may be efficient for the down-regulation rather than for the induction of autoreactive T cells and the amelioration of autoimmune disease.

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EXHIBIT E

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AN 1999:597309 PROMT
TI AutoImmune shares collapse on Colloral data in rheumatoid arthritis.
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TX US biotechnology firm AutoImmune saw its shares crash 74% on September 1 to close at $1.40 following the announcement that its oral tolerance drug Colloral (collagen) had failed in Phase III development. By the end of the trading week (September 3), the firm's stock was selling at a miserly $0.84, a nasty turnaround for a company which was riding high on the back of its oral tolerance technology a couple of years ago with stock being traded around the $14 mark.

AutoImmune says that Colloral will be dropped from development and the firm will "immediately reduce its headcount and other operating expenses to conserve resources as we evaluate our strategic options to maximize shareholder value." The company told the Marketletter that it plans to cut its workforce by 96%, downsizing to eight staff from 26 immediately and then to two employees by the end of the month.

In the 772-patient trial, Colloral was found to be safe but did not meet the primary endpoint, which the spokeswoman said was achieving statistical significance in three out of the "core-four" parameters (tender joints, swollen joints, physician's global assessment and patient global assessment). While AutoImmune says that "substantial improvements" from baseline were observed in each of these measurements, the placebo response was "much greater than previously observed." In fact, the spokeswoman noted that, although the data were not publicly available at present, the placebo response was two times higher than in previous studies of the drug. Full data may be presented at a forthcoming rheumatology meeting, and the firm is considering switching the focus of Colloral to a nutraceutical product.

When asked whether the trial could be designed differently, the spokeswoman told the Marketletter that it "was perfect." Financially, however, the firm cannot keep funding the clinical development of Colloral. AutoImmune had continued its clinical development of the drug even though earlier trials had failed to demonstrate strong data. Two years ago, the company revealed that two Phase II trials of Colloral in RA had failed to yield statistically significant results (Marketletter May 19, 1997). However, the firm decided to pursue Phase III development following an independent re-analysis by statisticians who concluded that the drug was significantly more effective than placebo (Marketletter September 15, 1997).

General expectations for the drug were not high, particularly following the earlier failure of another mucosal tolerance program, Myloral (myelin basic protein) for multiple sclerosis which performed no better than placebo in Phase III trials.

Yet some investors may see this a good buying opportunity, with analysts pointing out that the company has a decent cash position with few liabilities; as of June 30, the firm had cash and cash equivalents of almost $9.7 million and the spokeswoman added that once liabilities have been paid, this will be down to around $7 million.

Ideal opportunity to buy?

Furthermore, AutoImmune has a very strong intellectual property position and is still conducting a number of other trials which are funded

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externally. These include studies in new-onset type 1 diabetes (with Eli Lilly) and a pilot trial in chronic organ transplant rejection (results from both are due next year). Enrollment is continuing in a National Institutes of Health-funded long-term prevention study for type 1 diabetes.

Importantly, the firm also has an exclusive agreement with Teva Pharmaceutical for applications of AutoImmune's proprietary technology. The deal covers the development of an oral formulation of Teva's injectable multiple sclerosis drug Copaxone (glatiramer acetate) and an oral product for the treatment of myasthenia gravis, for which AutoImmune will receive milestone payments on product approval and royalties on any future sales. Teva is getting ready to start a Phase II/III trial of oral Copaxone with the first patient expected to be enrolled by year end, while the product for myasthenia gravis is also due to begin clinical development before the end of the year.

Despite speculation that the company's faith in the potential of inducing oral tolerance to antigens as a means of treating autoimmune disease may be misguided, AutoImmune says it still firmly believes in its technology. In a statement, the company said that "both basic and clinical research focused on enhancing the biological effect of (mucosal tolerance therapy) in patients will continue."

AutoImmune is currently assessing a number of different plans, including possible mergers and converting into a shell company while waiting for clinical data from its other ongoing trials to come through. The spokeswoman said that there has been interest from some firms in a merger, particularly as AutoImmune has such a strong IP position.

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EXHIBIT F

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Transplantation tolerance: The concept and its applicability

Dong VM, Womer KL, Sayegh MH. Transplantation tolerance: The concept and its applicability.
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Key words: allorecognition – costimulatory blockade – rejection – tolerance – transplantation

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Abstract: Recent advances have enabled researchers to induce tolerance in animal transplant models. Although it has been relatively easy to do so in rodents, it has been much more difficult to translate such strategies into primates. Understanding the cellular and molecular mechanisms of the alloimmune response has prompted the development of novel strategies that may obviate the need for immunosuppression in humans. Mechanisms of tolerance and promising new therapies, as well as the inherent difficulties in bringing them into clinical practice, are reviewed.

With improvements in surgical techniques, infectious disease therapy and overall medical care over the past few decades, manipulation of the immune response remains the major barrier to successful organ transplantation. The introduction of cyclosporin A into clinical use in the early 1980s (1) and the development of newer immunosuppressive drugs (2) have led to a significant reduction in acute rejection rates and improvement in short-term allograft survival. However, achieving long-term graft survival and overcoming chronic rejection remain difficult tasks (3, 4). Moreover, these drugs cause non-specific immunosuppression and result in an increased risk of infection, malignancy and cardiovascular disease. Therefore, the major goal of transplantation research is the development of strategies to induce donor-specific tolerance.

Tolerance has been defined as a state of specific immunologic unresponsiveness to the antigens of the graft in the absence of maintenance immunosuppression (5). However, it is now clear that active immunoregulatory mechanisms may be important in the development and maintenance of tolerance. Perhaps a more accurate definition of tolerance would be the absence of a destructive immune response against the graft in an immunocompetent host (6). *In vivo* criteria for donor-specific tolerance are the absence of acute rejection with prolongation of graft survival and acceptance of second test grafts from the original donor, while maintaining the ability to reject third party grafts. Unfortunately, investigators have only recently started to examine graft morphology and function in long-term surviving 'tolerant' animals. This is important, because long-term graft survival does not necessarily imply tolerance. In fact, tolerance should not only prevent acute rejection but also the alloantigen-dependent component of chronic allograft dysfunction, the major cause of late graft dysfunction and loss in solid organ transplantation (7).

Abbreviations: Ag, antigen; APC, antigen presenting cell; CD, cluster of differentiation; CTLA, cytotoxic T lymphocyte antigen; FasL, Fas ligand; gp39, glycoprotein 39; ICAM, intercellular adhesion molecule; ICOS, inducible co-stimulator; Ig, immunoglobulin; IL, interleukin; IL-2R, interleukin-2 receptor; IFN, interferon; LFA, leukocyte function associated antigen; MHC, major histocompatibility complex; TCR, T-cell receptor; Th, T helper; TNF, tumor necrosis factor.

* Co-first authors. This material was presented at the 3rd International Congress on Pediatric Transplantation, Boston, MA, USA, July 1998.

Billingham et al. in 1953 were the first to describe a state of actively acquired 'immunologic tolerance' in mice that had been injected *in utero* or during the neonatal period with bone marrow derived donor cells (8). Such animals would later accept an allograft taken from the same inbred strain from which the original cells were harvested, while maintaining the ability to reject third party strain grafts. Since these observations, extensive research has focused on developing strategies to understand the mechanisms of tolerance and to induce tolerance in adult animals. The ultimate goal would be to translate this research into large animals and eventually humans. Although it has been relatively easy to induce tolerance in rodents, it has proven to be much more difficult to translate such strategies into primates. Nonetheless, recent advances in our understanding of the cellular and molecular mechanisms of the alloimmune response have fueled the development of promising novel strategies that may be successfully translated into humans (5, 9, 10). The purpose of this review is to provide a brief overview of the mechanisms of tolerance, to describe some of the promising novel strategies that may be translated into humans, and finally to summarize the major problems we are facing in developing strategies to induce tolerance in humans.

Mechanisms of tolerance

Before discussing the current strategies designed to induce tolerance in the transplant recipient, it is worth reviewing the basic mechanisms of tolerance (11). Immunologic tolerance may be mediated by thymic deletional mechanisms (central tolerance) or be induced/maintained throughout the peripheral blood and extrathymic lymphoid tissue (peripheral tolerance) by at least three different mechanisms: deletion, anergy or regulation/suppression. These mechanisms will be reviewed as they relate both to self- and transplantation tolerance.

Self-tolerance

The physical elimination of certain T cells that interact with self-MHC molecules within the thymus plays an important role in the development of tolerance to self-antigens in the fetal/neonatal period (12). Phenotypic expression of T cells is determined by the sum of random TCR gene rearrangements (13). T-cell precursors originate in the bone marrow and migrate to the thymus where they initially fail to express the CD4 and CD8 T cell markers that are associated with maturity. These *double-negative* T cells then undergo proliferation and maturation and acquire both CD4 and CD8 T cell markers to become so-called *double-positive* cells. Through random TCR gene rearrangements, these thymocytes express TCRs with varying affinity for self-MHC + peptide.

Central to the mechanisms of self-tolerance is the avidity with which the TCR interacts with the self-MHC + peptide. This is determined by both the structure of the TCR for the antigen as well as the density of TCRs present on the T cell (14, 15). Thymocytes that either lack affinity or have very high affinity for the complex are negatively selected and undergo deletion by programmed cell death (apoptosis). Those thymocytes with low avidity for the complex are positively selected and become a set of T cells that function with self-MHC but lack sufficient autoreactivity to result in autoimmune disease. Some lower avidity TCRs may escape at this stage only to be deleted later with the increase in TCR expression that occurs with the transition from double positivity ($CD4^+/CD8^+$) to single positivity ($CD4^+/CD8^-$ or $CD4^-/CD8^+$) as thymocytes migrate from the cortex to the medulla of the thymus. Positively selected medullary thymocytes acquire MHC class II restricted helper ($CD4^+$) or MHC class I restricted cytotoxic ($CD8^+$) functions and migrate to the periphery as immunocompetent T cells. Between 95 and 99% of thymocytes are deleted in the thymus, with only the small remainder becoming mature T cells that migrate to the periphery. It is thought that autoimmunity arises when some autoreactive T cells escape thymic negative selection.

There are two types of thymic APCs: bone-marrow-derived macrophages/dendritic cells and epithelial cells (16). There is evidence that each type has a different function in the induction of self-tolerance and in T-cell repertoire selection. Positive selection is thought to be mediated by thymic epithelial cells, whereas negative selection is thought to be mediated by bone marrow-derived cells. Since the thymus involutes after puberty, clonal deletion may play only a minor role in the development of tolerance in the adult. However, cells that are important in the induction of thymic (central) deletional tolerance may still be functional. In addition, there is some evidence that T-cell death occurs in the periphery, assisting with the maintenance of self-tolerance and prevention of autoimmunity and possibly playing a role in transplantation tolerance.

Transplantation tolerance (Table 1)

Induction of tolerance in adults involves several mechanisms that are best classified as central or peripheral. Central tolerance involves *thymic deletional* mechanisms analogous to self-tolerance and can be induced in experimental animals by creating bone marrow chimeras (17). These chimeras are achieved by donor bone marrow infusion into a recipient treated with a myeloablative regimen such as total body irradiation. Donor APCs from this inoculum migrate to the thymus of the reconstituted animal, where they will be seen as *self*. Thereafter, the resultant animal will be specifically tolerant to donor alloantigen, presumably by deletion of alloreactive T cells in the thymus (negative selection).

Besides the potential complication of graft-versus-host disease, which can be prevented by T cell depletion of the bone marrow preparation, chimeric animals may have deficient T cell responses to nominal antigens. As previously mentioned, T cells are positively selected during normal maturation to recognize foreign antigens in association with the self-MHC molecules expressed on thymic epithelium. In these chimeras, however, the bone marrow-derived APCs are now of donor origin, whereas the thymic epithelial APCs remain of recipient origin. As a result, mature T cells will be positively selected to respond only to antigens presented by the recipient MHC molecule but not to antigen presented in association with donor-MHC on APCs derived from the donor bone marrow. Consequently, the T cell immune response is suppressed (17).

This problem can be overcome by creating mixed allogeneic chimeras by reconstituting the myeloablated animals with a mixture of both syngeneic plus allogeneic bone marrow (18). The reconstituted animals have a mixture of APCs derived from both recipient and donor and therefore have normal T cell function. Such a strategy has been successful in both rodents and non-human primates, in allo- as well as xeno-transplant models (17). However, the clinical applicability of such strategies is questionable because of the potential for major side-effects. Non-myeloablative regimens such as donor bone marrow infusion with administration of antilymphocyte serum or other immunosuppressive drugs have been used (19), although the mechanisms involved with such strategies may not be all deletional (20).

Mechanisms of peripheral tolerance generally involve *anergy* and/or *regulatory/suppressor cells*, although *peripheral deletional* mechanisms probably also play a role. One possible mechanism leading to tolerance that has been described with donor bone marrow infusion strategies is *microchimerism* (21, 22). Microchimerism is the persistence of small numbers of donor-derived cells in the recipient and has been reported to be associated with long-term acceptance of allografts in experimental animals as well as in humans (23). However, it has not yet been established whether the persistence of donor cells is actually responsible for the induction or maintenance of tolerance. The observation by Fraser et al. that the donor bone marrow can be replaced with recipient marrow transfected with donor MHC indicates that microchimerism is not necessary for graft acceptance and suggests that a supply of donor antigens for presentation by the indirect pathway of allorecognition (i.e. by recipient APCs) may be tolerogenic (24). The distinction between the indirect and direct pathways will be discussed later in more detail.

Veto cells are cells with a unique phenotype that have been shown to inactivate/delete alloreactive T cells. These cells have been described in some transplantation models involving donor bone marrow infusion (21, 22), although the mechanisms of how T cells recognize the veto cell leading to T cell inactivation/deletion remain unknown. Furthermore, whether veto cells mediate the observed association between graft acceptance and microchimerism has not been established.

Anergy is a state of functional inactivation in which antigen-specific T lymphocytes are present but unable to respond. Unresponsiveness can be assessed *in vitro* by failure of proliferation and cytokine production (25) and *in vivo* by failure of clonal expansion (26, 27). Two types of anergy-related mechanisms have been described: 1, T cell anergy, which may be reversed by exogenous cytokines such as IL-2 (28, 29); and 2, *dense* anergy, which is not reversed by cytokines (30). Recent evidence also indicates that anergy can be accompanied by variable degrees of deletion and that anergic T cells may become apoptotic (31). From the standpoint of clinical applicability, strategies that promote peripheral deletion of anergic T cells are more desirable. In this regard, there has been recent interest in studying the role and mechanisms of activation induced cell death in induction and maintenance of transplantation tolerance (32, 33).

It is now clear that T cells require two distinct signals for full activation. The first signal is provided by the engagement of the TCR with the MHC + peptide complex on APCs, and the second *costimulatory* signal is provided by

engagement of one or more T cell surface receptors with their specific ligands on APCs. TCR ligation in the absence of additional signals provided by the MHC-expressing APC results in antigen-specific unresponsiveness or anergy (34). While several receptor–ligand interactions have been suggested to provide costimulatory signals to T cells (CD2: LFA-3, LFA-1: ICAM-1), the best characterized costimulatory signal involves binding of the T cell surface molecule CD28 to either of its ligands, B7-1 or B7-2, expressed on the surface of professional bone marrow derived APCs (see Fig. 1).

The uniqueness of this costimulatory pathway has been demonstrated clearly in studies indicating that signaling through CD28 prevents T cell anergy and death induced by TCR signaling alone (35, 36). CD28 has a high degree of homology (32% identity at the amino acid level) to another gene called CTLA4 (37). Unlike CD28, which is expressed on resting T cells, CTLA4 appears to be expressed on the cell surface only after initial T cell activation. CTLA4 appears to downregulate immune responses by binding with high affinity to its B7 counter-receptors (38, 39). CTLA4 gene knockout mice show a variety of lymphoproliferative disorders and early death (40), while administration of blocking anti-CTLA4 monoclonal antibodies (to block negative T cell signaling) worsens autoimmune disease (41) and prevents induction of peripheral tolerance (42), implying a critical physiologic role for CTLA4 in terminating T cell responses (43).

Another means by which tolerance is regulated is through the induction of specific regulatory or *suppressor* cells. These cells have been demonstrated *in vitro* by suppressor assays as well as *in vivo* by adoptive transfer experiments that may lead to a state of 'infectious' tolerance (44, 45), whereby T cells from a tolerant animal may actively transfer tolerance to a naive animal. Although suppressor phenomena have been clearly demonstrated, the suppressor cells themselves have been difficult to clone and, hence, to characterize. In addition, the mechanism of action of suppressor cells is still poorly understood, although recent data suggest that the tolerogenic effects may be mediated by suppressive cytokines (46).

A related area which may explain the role of these regulatory cells in tolerance is the controversial T_H1/T_H2 paradigm (47–50). It has been recognized that $CD4^+$ T cells can be subdivided into T helper 1 (T_H1) and T helper 2 (T_H2) subsets that are classified according to the profile of cytokines they secrete. T_H1 cells secrete IL-2 and IFN-γ and predominately mediate cellular immune responses, while T_H2 cells secrete IL-4, IL-5, IL-10 and IL-13 and regulate humoral immune responses. T_H1 cytokines have been shown to be upregulated in rejecting allografts, while T_H2 cytokines have been shown to be expressed in 'tolerant' grafts (51, 52). Such a state of immune deviation towards predominately T_H2 cell function has been associated with tolerance in models of transplantation, although the causality of such an association has not been proven. Indeed, recent evidence in specific cytokine gene knockout animals showing that T_H1 knockout mice can reject a graft (53) and that T_H2 knockout animals can be tolerized (54) illustrates the complexity of the system.

Novel clinical strategies

The major shortcomings of modern transplantation medicine are the consequences of lifelong immunosuppression. It would be more desirable to achieve a state of immunologic tolerance, which would obviate the need for immunosuppression. This challenge is heightened by the fact that immunosuppressive agents may actually abrogate the induction of tolerance (55–57). Nevertheless, there are a number of promising strategies currently being investigated.

Intrathymic tolerance

The initial studies in the 1960s of intrathymic inoculation of soluble antigen into adult rats demonstrated antigen-specific systemic T cell unresponsiveness. It is now recognized that this unresponsiveness is probably achieved via anergy and cell deletion (apoptosis) (58). Intrathymic injection of antigen (Ag) induces Ag-specific tolerance in several experimental autoimmune and transplantation models. However, the clinical applicability of thymic injection of alloantigen still requires further investigation (59, 60).

Central tolerance inducing strategies that manipulate T cell events in the thymus have involved lethal or sublethal myeloablation followed by immune response reconstitution with a combination of donor and recipient bone marrow, as mentioned earlier (61). Another approach uses transfected recipient bone marrow cells with donor MHC, obviating the need for donor bone marrow cells. In an effort to reduce the toxic irradiation dose to large animals, synergistic therapies such as polyclonal/monoclonal antilymphocyte antibodies, cyclophosphamide or costimulatory blockade have been or are being tested. This approach allows for host bone

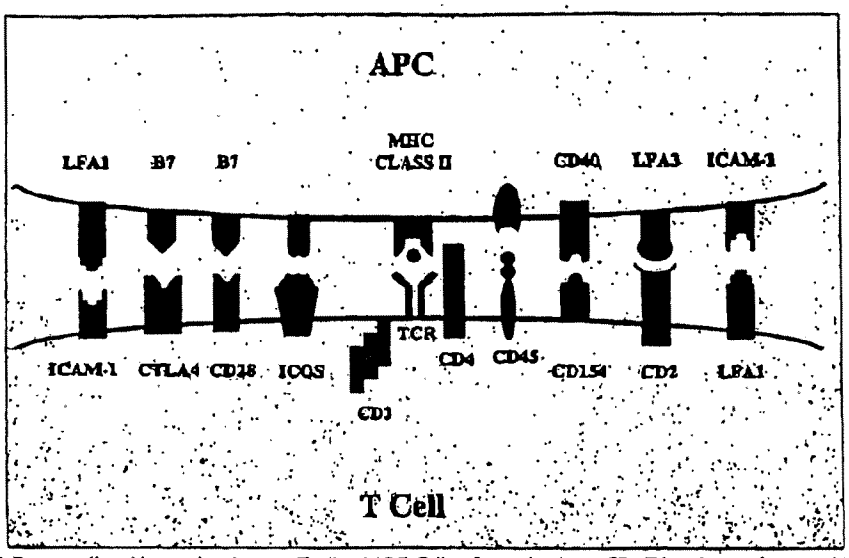

Fig. 1. Receptor–ligand interactions between T cell and APC. Cell surface molecules on CD4 T lymphocytes interact with their respective ligands on the APC. Interactions between these molecules are important in cell–cell adhesion and/or transmission of signals (including costimulatory signals) to immune cells.

marrow suppression with minimal side-effects, resulting in stable mixed chimeric animals. These models have shown promise in non-human primates as a preclinical strategy (62).

Donor bone marrow infusion

The use of donor bone marrow augmentation utilizes aspects of both central and peripheral tolerance (23). Donor bone marrow is injected into patients at the time of solid organ transplantation, with subsequent low level persistence of donor cells leading to a microchimeric state. As discussed previously, long-term allograft function in humans and animals is often associated with persistence of donor-derived hematopoietic cells in the recipient. However, it is unclear whether donor cells persist due to immunosuppression or if they are themselves responsible for prolonged long-term graft survival. Barber et al. examined the use of Minnesota antilymphocyte globulin and subsequent transfusion of donor-specific bone marrow in 57 cadaveric renal allograft recipients. When compared with controls, donor-specific bone marrow infusion was associated with improved allograft survival (63). More recently a clinical trial looked at the effect of timing and dose of peripheral donor bone marrow cell infusion on graft and patient survival after liver transplantation (64). As with the renal allograft recipients, graft survival was significantly improved in the treatment group. The optimal utility of bone marrow infusion/augmentation has not been defined in multicenter, randomized clinical trials.

Immunomodulatory peptides

Observations that some peptides bound to MHC molecules are derived from MHC sequences themselves has prompted the study of the immunoregulatory properties of MHC derived peptides. In transplantation, two pathways of allorecognition are recognized (10, 65–67). The direct pathway involves recipient T cells recognizing intact allo-MHC molecules complexed with peptide on the surface of donor cells. The indirect pathway involves recipient APCs processing and presenting alloantigens (mainly allo-MHC peptides) to recipient $CD4^+$ cells (see Fig. 2). In the latter situation, T cell responses to an alloantigen may be limited to one or only a few dominant peptide determinants. Therefore, tolerizing to these peptide determinants alone may then successfully inhibit at least indirect T cell alloresponses (68).

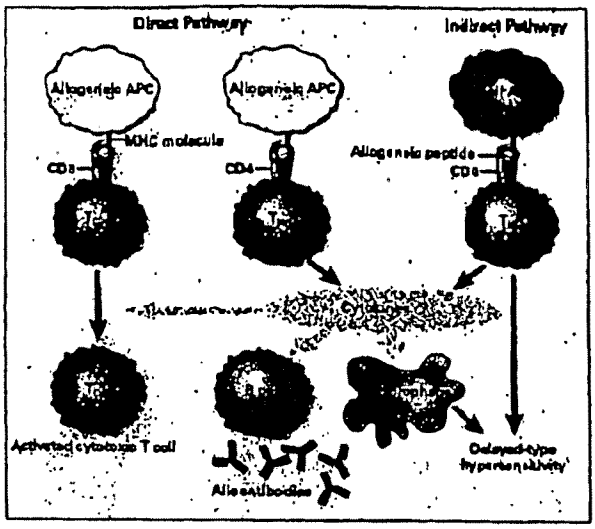

Fig. 2. Pathways of recognition of allogeneic MHC molecules and mechanisms of graft rejection. Graft rejection is usually initiated by CD4 helper T cells (T_H) that bind peptides in complexes with MHC class II molecules on antigen-presenting cells. In the direct pathway, an MHC molecule on a foreign (allogeneic) cell, such as an APC, binds to the helper T cell. In the indirect pathway, the foreign MHC molecule is processed into peptides that are presented to the helper T cell by one of the recipient's own APCs. In either scenario, activated CD4 helper T cells proliferate and secrete cytokines that serve as growth/activation factors for CD8 cytotoxic cells (T_C), B cells and macrophages. These cells in turn cause graft destruction by target cell lysis, antibody production and delayed-type hypersensitivity reactions, respectively. [Reprinted with permission: N Engl J Med 1998;338:1813–21. © 1998 Massachusetts Medical Society. All rights reserved.]

Strategies designed to induce allogeneic unresponsiveness using peptides derived from both polymorphic and non-polymorphic portions of class I and II MHC have been studied (69). Boytim et al. were able to block allo-non-specific induced T cell proliferation with a synthetic peptide corresponding to the alpha helical region of HLA-DQ by interrupting cell-cycle progression (70). Similarly, Murphy et al. were able to alter T cell alloimmune responses *in vitro* with synthetic peptides derived from a highly conserved region of the class II MHC alpha chain through the induction of apoptosis (71). *In vivo* studies have demonstrated reduced delayed-type hypersensitivity responses to a mixture of polymorphic class II MHC allopeptides in peptide immunized rats with oral administration of the peptide mixture prior to immunization (72). Since indirect allorecognition may play a role in chronic rejection, synthetic MHC peptides may also be used in the future to develop predictive assays that link T cell activity to the subsequent risk of developing chronic rejection post-transplant (73, 74). Whether tolerizing to MHC peptides, by oral administration for example, will have an impact on delaying progression of chronic rejection remains to be seen.

Other therapies

The targeting of T cell epitopes by monoclonal antibodies is currently undergoing intense investigation. The humanized monoclonal antibodies, daclizumab and basiliximab, are targeted against the 55Kd alpha subunit of the IL-2 receptor (2) and have recently been approved by the FDA for use as induction therapy in clinical renal transplantation (75). However, their role in the induction of tolerance has not yet been established.

There has been an increased understanding of the molecular interactions between the T cell and APC, as discussed previously. Illustrated in Fig. 1 are some of the better characterized receptor-ligand couplings (2). Antibodies directed against some of these cell surface molecules have been used in experimental small animal models to induce tolerance (76). The clinical applicability of some of these strategies is currently being tested.

Newer approaches still in their infancy include gene-targeted therapies (77). For example, it has been demonstrated in the mouse diabetic model that FasL expressed in syngeneic myoblasts, made by transfection of myoblasts of recipient origin, can protect allogeneic islets of Langerhans from rejection, presumably through Fas/FasL-induced apoptosis of reactive T cells (78). In clinical transplantation, gene therapy may enable immunomodulatory agents to be expressed in the graft, thereby overcoming the difficulties of systemic immunosuppresion (2).

T-cell costimulatory blockade

Although there are several experimental strategies that may hold promise for the induction of

Table 1. Mechanisms of transplantation tolerance

Clonal deletion
Central (thymic)
Peripheral (passive cell death or activation induced cell death)
Microchimerism (possible role of veto cells)
Clonal energy
Regulatory cells (infectious tolerance)

transplantation tolerance in humans (Table 2), T-cell costimulatory blockade is perhaps the most promising and will likely be tested clinically in the near future. Ligation of CD28 by B7-1 or B7-2 is blocked by CTLA4Ig, a recombinant fusion protein that contains the extracellular domain of soluble CTLA4 fused to an Ig heavy chain (79). CTLA4Ig binds to both B7-1 and B7-2 and acts as a competitive inhibitor of CD28 binding to B7-1 and B7-2, resulting in T cell anergy *in vitro* (80, 81) (see Fig. 3). T cell costimulatory blockade by systemic administration of CTLA4Ig has been very effective in preventing experimental acute rejection, prolonging graft survival and inducing specific tolerance in some transplantation models (10). Recent studies from our laboratory have demonstrated that CD28-B7 T cell costimulatory blockade by CTLA4Ig prevents development of chronic rejection in rat models of cardiac (82) as well as renal (83) transplantation.

More interestingly, CD28-B7 blockade late after acute injury interrupts progression of chronic rejection in an experimental chronic renal allograft rejection model (84). The exact mechanisms mediating induction of tolerance by CD28-B7 T cell costimulatory blockade *in vivo* remain unclear (10). It has been suggested that CTLA4Ig induces T cell anergy and prevents expansion of antigen-specific T cells *in vivo* (26). In the mouse heart transplant model, the induction of unresponsiveness requires an intact CTLA4 negative signaling pathway (85). Our studies in the rat acute renal allograft rejection model indicate that systemic tolerance induced by the administration of CTLA4Ig is associated with selective inhibition of Th1 and sparing of Th2 cytokines in the target organ (51). However, as indicated earlier, recent data in cytokine gene knockout animals highlight the complexity of studying the role of cytokines in allograft rejection and tolerance. To further complicate the picture, inducible costimulator (ICOS), a proposed third member of T cell specific cell surface receptors, CD28 and CTLA4, has recently been identified (86) (see Fig. 1) What is interesting is that unlike constitutively expressed CD28, ICOS must be induced *de novo* on the surface of the T cell and does not upregulate the production of IL-2, but superinduces the synthesis of IL-10. However, the role of ICOS in graft rejection and/or tolerance remains to be determined.

Recently, there has been great interest in studying the role of CD40 and its ligand, CD40L, in the process of allograft rejection and tolerance (10) (see Fig. 4). CD40, a member of the TNF receptor family, is expressed on B cells and other APCs, including dendritic cells and endothelial cells (87). CD40L (a member of the TNF family, also known as gp39), is expressed early on activated T cells (87). Binding of CD40L to CD40 is critical in providing T-cell help for B-cell Ig production and class switching (88); a defect in CD40L is responsible for the hyper-IgM syndrome in humans (89).

The role of CD40L in T-cell activation has been uncertain. Studies using CD40L knockout mice have demonstrated an inability of CD40L-deficient T cells to undergo effective clonal expansion (90, 91). It has been questioned, however, whether CD40L acts directly to transduce a costimulatory signal to the T cell or indirectly, since ligation of CD40 on APCs is a strong inducer of B7 expression (92, 93). Therefore, CD40L on the T cell might merely serve to induce CD28-ligands or other costimulatory molecules on APCs. Several studies in

Table 2. Novel experimental approaches

Intrathymic tolerance
Bone marrow infusion/ augmentation and reconstitution
Class I and class II MHC-derived peptides and MHC proteins
Costimulatory T cell activation pathway blockade: CTLA4Ig, anti-B7 antibodies and humanized anti-CD40L
Gene targeted therapies: Fas, transfection, cytokines and immunomodulatory agents

Table 3. Transplantation tolerance: why is it difficult to translate animal studies to humans

Dependence on genetically defined inbred strains of small animals
Pathogen-free small animal facilities
Differences in expression of class II MHC and/or costimulatory molecules on endothelial cells and T cells between species
Involution of adult human thymus
Effect of conventional immunosuppression

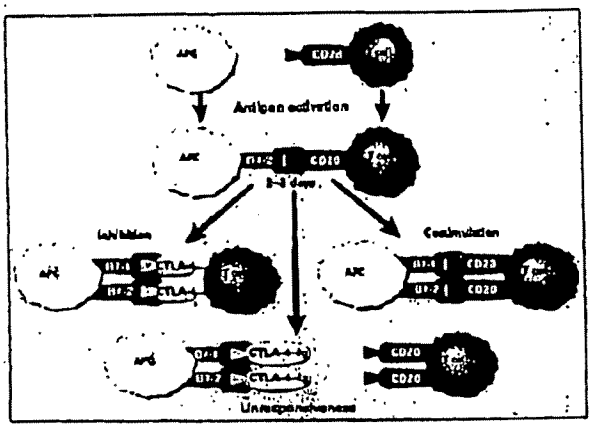

Fig. 3. Functions of CD28, B7-1, B7-2 and CTLA-4 molecules. Resting T cells express CD28, but concurrently resting APCs do not express B7 molecules. Within six h of activation, B7-2 is expressed by APCs and becomes available for binding to CD28, transmitting a costimulatory signal to the T cell. By 48–72 h after activation, APCs also express B7-1, while T cells express the CTLA-4 inhibitory receptor. Both B7-1 and B7-2 may bind either to CD28 or CTLA-4, providing continued costimulation or a new inhibitory signal, respectively. Since CTLA-4 has a higher affinity for B7 molecules than CD28, its inhibitory interaction ultimately prevails, leading to immune response termination. The fusion protein CTLA4Ig may compete with CD28 and CTLA-4 for B7 binding, thus blocking costimulatory interactions. [Reprinted with permission: N Engl J Med 1998;338:1813–21. © 1998 Massachusetts Medical Society. All rights reserved.]

experimental transplantation models indicate that CD40L blockade can prevent acute rejection and prolong allograft survival (52, 94, 95). Larsen et al. (55) addressed the potential for synergy between B7 and CD40L blockade by demonstrating that inhibition of these two pathways leads to prolonged allogeneic mouse skin and cardiac allograft survival. An interesting finding in this study was that only combination therapy with both anti-CD40L and CTLA4Ig was able to prevent graft arteriosclerosis and fibrosis (chronic rejection). Recent studies in islet (96) as well as renal (97) primate transplant recipients indicate the efficacy of T cell costimulatory approaches in preclinical models and provide the rationale to develop such strategies in human organ transplant recipients.

One of the interesting observations in several of the experimental studies with T cell costimulatory blockade is the fact that administration of donor antigen appears to synergize with CTLA4Ig or anti-CD40L in promoting long-term graft survival (52, 95, 98, 99). In some models, the administration of donor cells was necessary to prevent the development of chronic rejection (56, 100). More interestingly, a recent study by Wekerle et al. showed that induction of chimerism with donor bone marrow and T cell costimulatory blockade results in delectional tolerance. This strategy may have applicability

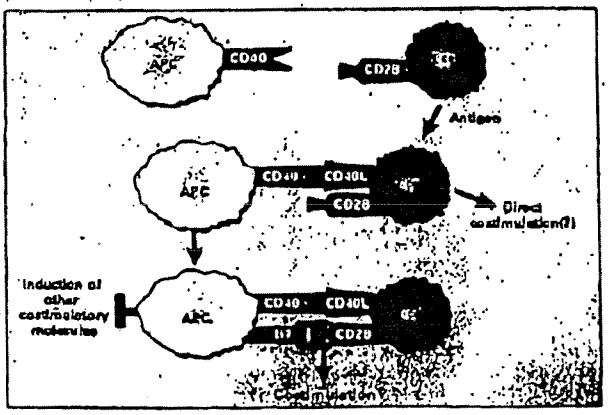

Fig. 4. The CD40L-CD40 pathway and its association with the CD28-B7 pathway. Resting APCs, that include B cells, macrophages and dendritic cells express CD40. When activated, T cells express CD40 ligand. The CD40L-CD40 interaction is important in providing T cell help to prevent apoptosis as well as to induce immunoglobulin production and isotope switching. Activation of CD40 on APCs provides a signal for B7 induction, especially B7-2. CD40 ligand may act in T cell costimulation by directly providing costimulation, by inducing B7, or by inducing other costimulatory ligands. [Reprinted with permission: N Engl J Med 1998;338:1813–21. © 1998 Massachusetts Medical Society. All rights reserved.]

to humans, because it may allow clinical translation of the bone marrow chimerism approach, without myeloablation or T cell depletion of the host (19).

After a decade of laboratory studies, CTLA4Ig has now been used in the clinical arena. Abrams and others have recently published the results of a phase I clinical trial describing the immunosuppressive effects of CTLA4Ig in the T cell mediated autoimmune disease, psoriasis vulgaris (101). This timely initial trial serves to underscore the possible application of costimulatory blockade to various clinical diseases, including transplant rejection (102).

Clinical tolerance

Despite the fact that it has been relatively easy to induce true tolerance in small experimental animals, translating these studies into larger animals and humans has been much more difficult to achieve. Some of the hurdles that may explain this dilemma are summarized in Table 3. Even if we have the ideal strategy to use in humans, the lack of reliable predictive assays for rejection or tolerance still does not allow us to know if a patient is truly tolerant so that immunosuppressive agents may be withdrawn. Rechallenging a transplant recipient with a second test graft to prove tolerance is not feasible. Therefore, we must define achievement of transplantation tolerance in clinical, immunologic and molecular terms. Once we accomplish this task, transplantation tolerance will no longer be an elusive goal.

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EXHIBIT G

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Rapid communication

No effect of oral insulin on residual beta-cell function in recent-onset Type I diabetes (the IMDIAB VII)

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Abstract

Aims/hypothesis. Induction of tolerance to insulin is achievable in animal models of Type I (insulin-dependent) Diabetes mellitus by oral treatment with this hormone, which can lead to prevention of the disease. In the Diabetes Prevention Trial of Type I diabetes (DPT-1), oral insulin is given with the aim of preventing disease insurgence. We investigated whether if given at diagnosis of Type I diabetes in humans, oral insulin can still act as a tolerogen and therefore preserve residual beta-cell function, which is known to be substantial at diagnosis.
Methods. A double-blind trial was carried out in patients (mean age ± SD: 14 ± 8 years) with recent-onset Type I diabetes to whom oral insulin (5 mg daily) or placebo was given for 12 months in addition to intensive subcutaneous insulin therapy. A total of 82 patients with clinical Type I diabetes (< 4 weeks duration) were studied. Basal C peptide and glycated haemoglobin were measured and the insulin requirement monitored every 3 months up to 1 year. Insulin antibodies were also measured in 27 patients treated with oral insulin and in 18 patients receiving placebo at the beginning of the trial and after 3, 6 and 12 months of treatment.
Results. The trial was completed by 80 patients. Overall and without distinction between age at diagnosis, at 3, 6, 9 and 12 months baseline mean C-peptide secretion in patients treated with oral insulin did not differ from that of those patients treated with placebo. In patients younger than 15 years a tendency for lower C-peptide values at 9 and 12 months was observed in the oral insulin group. Insulin requirement at 1 year was similar between the two groups as well as the percentage of glycated haemoglobin. Finally, IgG insulin antibodies were similar in the two groups at each time point.
Conclusion/interpretation. The results of this study indicate that the addition of 5 mg of oral insulin does not modify the course of the disease in the first year after diagnosis and probably does not statistically affect the humoral immune response against insulin. [Diabetologia (2000) 43: 1000–1004]

Keywords Type I diabetes, oral insulin, insulin antibodies, prevention.

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Abbreviations: DPT-1, Diabetes Prevention Trial of Type I diabetes; IA, insulin antibodies

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The use of insulin, given either subcutaneously or orally, in subjects at risk for Type I diabetes has been recently introduced in the Diabetes Prevention Trial of Type I diabetes (DPT-1) trial, a large multinational trial in the United States, with the aim of preventing the destruction of beta cells and the clinical onset of the disease [1]. The rationale for the use of insulin in these patients is to induce beta-cell rest and/or tolerance to the hormone and its peptides [2] which are thought to be important targets of the autoimmune response leading to beta-cell destruction [3]. In ani-

Table 1. Baseline clinical characteristics and metabolic control at entry of the trial in the two groups of patients

	Oral insulin	Placebo
Number of patients	46	36
Sex (number of males)	25	17
Age (years ± SD)	14.1 ± 7.9	13.8 ± 7.6
Duration of symptoms before diagnosis (days)	34.5 ± 34.6	38.8 ± 42.5
Blood glucose at diagnosis (mmol/l)	20.7 ± 9.4	22.1 ± 8.3
Insulin dose (U · kg^{-1} · day^{-1}) ± SD	0.63 ± 0.3	0.55 ± 0.3
Glycated haemoglobin (%) ± SD	9.9 ± 0.5	9.0 ± 0.5
Basal C peptide (nmol/l) ± SD	0.23 ± 0.3	0.17 ± 0.15

Values between groups are not statistically different

mal models, this approach was shown to be effective in halting the process leading to Type I (insulin-dependent) diabetes mellitus [4]. Furthermore, oral insulin was able to reduce the extent and modify the type of lymphocytic infiltration in the pancreas of susceptible mice [5].

The International Diabetes Immunotherapy Group suggested that approaches to prevent Type I diabetes should first be tested in recent-onset Type I diabetic patients and, if effective, applied to pre-diabetic people [6]. In our study we evaluated the effects of oral insulin treatment at clinical onset of Type I diabetes, which could aid in reducing the further destruction of beta cells that generally occurs within the first 12 months after diagnosis. In patients with recent-onset Type I diabetes, simultaneous treatment with subcutaneous and oral insulin might have considerable effects, as the former could improve metabolic control and the latter induce tolerance. As disturbances in the gut immune reactivity could be relevant in the pathogenesis of Type I diabetes [7], induction of oral tolerance with a specific antigen, such as insulin, could be appropriate for this disease. It is therefore the aim of this double-blind randomized trial to find out whether treating patients who have recent-onset Type I diabetes with oral insulin in addition to identified subcutaneous insulin therapy [8] could improve metabolic control, as measured by glycated haemoglobin value, insulin dose and C peptide concentration. The effects of such treatment on the rate of spontaneous clinical remission (suspension of insulin therapy) and on the extent of humoral immune response against insulin were also evaluated.

Subjects and methods

Selection of patients. Patients with recent-onset Type I diabetes ($n = 82$) were recruited by 8 participating centres of the IMDIAB Group and 1 affiliated centre. Each centre contributed with nearly equal numbers of patients to the study. Inclusion criteria were the following: (1) diagnosis of the disease according to the World Health Organisation (WHO) criteria, with age at presentation between 5 and 35 years, (2) duration of clinical disease (since the beginning of insulin therapy) less than 4 weeks, (3) no medical contra-indications (including pregnancy) or any other major chronic disease, (4) willingness and capability to participate in regular follow-up.

Patients' baseline clinical characteristics and metabolic control at entry of the trial are shown in Table 1.

Study design and treatment protocol. The study was endorsed by the Italian Ministry of Health and approved by the central ethics committee at the Gemelli Policlinic, The Catholic University of the Sacred Heart, Rome. After informed consent had been obtained and baseline measurements completed, a permuted-block design was used to blindly assign patients to each of the two treatment groups. A random number table was adopted with a prepared list and a randomization code was assigned to each participating centre. Of the patients 46 received 5 mg daily of oral insulin and 36 placebo. Oral treatment began within 4 weeks of diagnosis in both groups and lasted 12 months. All patients also received intensive subcutaneous insulin therapy as soon as possible after diagnosis to optimize metabolic control and maintain blood glucose concentrations as near to normal as possible (see below).

Guidelines for insulin therapy. All participating centres used the same treatment protocol as in our previous IMDIAB trials [9, 10] based on the following rules: if pre-prandial blood glucose values were below 6.5 mmol/l, the insulin dose was decreased by 10%; if blood glucose concentrations were consistently below 4.5 mmol/l for more than 3 days the insulin dose was decreased by 20%. Insulin therapy was not discontinued unless 2-h postprandial blood glucose concentrations measured at home were consistently below 7.5 mmol/l. Patients with blood glucose above 10 mmol/l received a 10% increase in insulin dose or had their insulin regimen modified. Frequent telephone consultations were arranged with patients to adjust the insulin dose as required.

Investigations and follow-up. Patients included in the study were followed up by the staff of the centre where they were enrolled. Patients were started on a 55% carbohydrate diet and received three to four injections daily of regular plus intermediate insulin. Each patient recorded capillary glucose concentration at fasting and before and after meals daily, for a total of at least 20 weeks. The subcutaneous insulin dose was adjusted to obtain near-normal blood glucose concentrations.

Patients were examined weekly for the first month of therapy and then monthly by the same team of physicians in each participating centre. Drug toxicity was evaluated at follow-up visits, by liver and renal function tests and total blood count. Glycated haemoglobin (HbA_{1c}) (normal range 4–7%) was measured every 3 months by a column assay (Bio-Rad, Milan, Italy), and basal C peptide concentration was evaluated after euglycaemia was achieved before entry into the trial, and at 3-monthly intervals for 1 year thereafter. C peptide concentra-

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Table 2. Metabolic outcomes during follow-up

	Oral insulin	Placebo
Number of patients	44	36
Insulin dose (U/kg) ± SD		
3 months	0.44 ± 0.3	0.39 ± 0.2
6 months	0.48 ± 0.3	0.43 ± 0.2
9 months	0.54 ± 0.3	0.52 ± 0.3
12 months	0.61 ± 0.2	0.58 ± 0.3
Glycated haemoglobin (%) ± SD		
3 months	6.2 ± 1.8	5.8 ± 1.5
6 months	6.5 ± 1.5	6.3 ± 1.5
9 months	7.1 ± 1.6	7.1 ± 1.5
12 months	7.6 ± 1.3	7.1 ± 1.5
Basal C peptide (nmol/l) ± SD		
3 months	0.30 ± 0.2	0.30 ± 0.2
6 months	0.30 ± 0.2	0.30 ± 0.2
9 months	0.20 ± 0.2	0.25 ± 0.2
12 months	0.17 ± 0.2	0.22 ± 0.2

Values between the two groups are not statistically different for insulin dose, HbA_{1c}, C peptide concentration

tion was measured by radioimmunoassay, using a commercially available kit (Bio-Rad). The normal range of fasting C peptide established in 150 control subjects, 71 females and 79 males, aged 5–40 years, median 18 years, with no family history of Type I diabetes was 0.35–1 nmol/l with intracoefficients and intercoefficients varying between 10% and 15%, respectively.

Insulin antibodies. Insulin antibodies (IA), expressed as a concentration of units/5 µl serum, were measured in serum samples drawn from 27 patients treated with oral insulin and 18 receiving placebo at the beginning of the trial and after 3, 6 and 12 months of treatment and stored at −20 °C. A modification of the micro-radio-binding assay of Williams [11] was used as described previously [12]. The threshold and 99th centile of 97 control subjects, 51 females and 46 males, aged 2–48 years, median 21 years, with no family history of Type I diabetes, was calculated at greater than 4.4 insulin antibody units.

Evaluation of response to therapy. Response to therapy was monitored throughout the study by investigating the occurrence of clinical (complete) remission defined, according to the recommendations of the International Diabetes Immunotherapy Group (IDIG), as restoration of normal fasting and postprandial blood glucose concentration without any insulin treatment for more than 2 weeks [6]. Moreover, metabolic control (C peptide, HbA_{1c} and insulin dose) was evaluated at 3-monthly intervals.

Sample size and statistical analysis. The number of patients to be included in the study was calculated from an analysis of results of trials published in the past (courtesy of IDIG Registry). Setting alpha (probability of a type I error) equal to 0.05 and beta (probability of a type II error) equal to 90%, the required sample size was 74 patients for a two-sided test. To ensure the appropriate sample size, 82 patients were recruited to allow for drop outs.

Results obtained in the different treatment groups were analysed blind by a team of statisticians. Differences in clinical remission proportions between patient groups were evaluated by the one-sided Fisher's exact probability test. For the analysis of the integrated measures of metabolic control (C peptide, HbA_{1c} and insulin dose), an analysis of variance was done; for measuring differences between groups at different time intervals, the Mann-Whitney U test was used.

For the analysis of antibody results, median antibody values in the two groups at each time point were compared using the Mann-Whitney U test, whereas proportions of patients with IA at each time point were compared using Fisher's exact probability test.

Results

Recruitment lasted 1 year. There were no significant differences between the two groups of patients in baseline clinical characteristics and metabolic control at the time of enrollment (Table 1). None of the patients suffered from any other autoimmune disease.

Dropouts. Only two patients withdrew from the study and this was because of poor compliance.

Metabolic data. Clinical remission was observed in one patient (lasting 3 months) in the oral insulin group and one patient (lasting 8 months) in the placebo group. Insulin requirement was significantly reduced in all patients after 3 months of treatment compared with the beginning of the trial but the patients treated with oral insulin and placebo did not differ in this respect (Table 2). The subcutaneous insulin dose required to obtain optimal metabolic control was similar in the two groups at 6, 9, and 12 months. Basal C-peptide secretion over 1 year of follow-up had a similar pattern in both groups of patients, with an initial increase (compared with diagnosis), followed by a steady decrease which was slightly more pronounced in the oral insulin group. When age at diagnosis was taken into account, insulin dose, HBA_{1c} values and C-peptide concentrations in patients older than 15 years ($n = 28$) were not different at the beginning of the trial between oral insulin ($n = 16$) and placebo-treated ($n = 12$) patients and did not change thereafter. In patients younger than 15 years ($n = 52$), C-peptide concentrations after 9 and 12 months tended to decline more in the oral insulin ($n = 28$) than in the placebo group ($n = 24$), although the difference between the two groups did not reach statistical significance (Fig. 1). Good metabolic control was achieved by all patients, as shown by the rapid decline in HbA_{1c} values after diagnosis, which persisted until the end of the study. Finally, no adverse effects were noted in patients receiving either oral insulin or placebo.

Insulin antibodies. Insulin antibodies were detectable at disease onset in 17 out of 27 (63%) patients receiving oral insulin and 8 out of 18 (44%) receiving placebo; the humoral response against insulin increased during the study in 23 of the patients receiving oral insulin and 15 of those treated with placebo. No dif-

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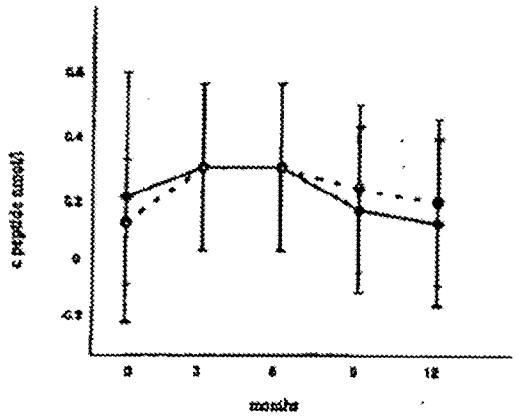

Fig. 1. Basal C-peptide concentration (mean ± SD) in patients younger than 15 years treated with oral insulin ($n = 28$, —●—) and placebo ($n = 24$, —●—). Values were lower at 9 and 12 months in the patients treated with oral insulin, however they were not statistically different from those patients treated with placebo

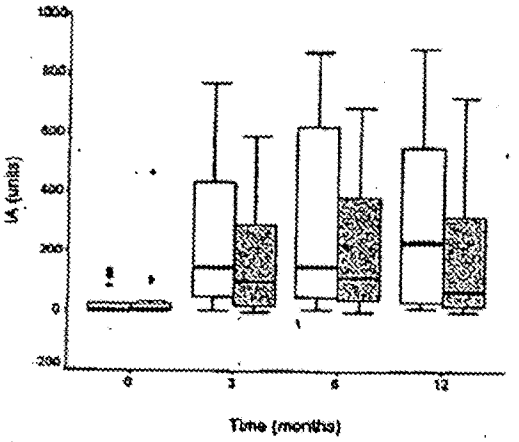

Fig. 2. Box-whisker plot of insulin antibodies measured at various time points in patients treated with oral insulin (filled boxes) or placebo (open boxes). Values between the two groups were not statistically different at any time point

ferences were observed between the two groups in the proportion of patients with IA or in the median insulin antibody concentrations at all time points, although a trend towards lower antibody concentrations was evident in the group treated with oral insulin after 3, 6 and 12 months of treatment (Fig. 2). When antibody results were analysed according to age at diagnosis (15 years), levels of IA after 3, 6 and 12 months were higher in younger patients ($n = 32$) than in older subjects ($n = 13$) ($p < 0.02$ at all time points) but no significant differences were observed between patients treated with oral insulin and those treated with placebo when subdivided by age (data not shown).

Discussion

This double-blind trial with oral insulin in patients with recent-onset Type I diabetes was designed to assess whether the addition of oral insulin at the time of clinical diagnosis could maintain or even improve the residual beta-cell function which is usually detectable in these patients. Oral insulin had no effect on residual beta-cell function, as assessed by C-peptide secretion. Furthermore, patients treated with oral insulin who were younger than 15 years at diagnosis showed a tendency for a more pronounced decline of basal C-peptide concentrations 9 and 12 months after diagnosis compared with patients matched with them for age but treated with placebo, although this difference was not statistically significant. We did not measure stimulated C peptide but limited the investigation to baseline C peptide concentrations. These were measured under strict and controlled conditions of fasting blood glucose less than 180 mg/kg at the time of sampling (if blood glucose concentration was higher sampling for C peptide was postponed). The night before the test patients were also advised to have a light meal and avoid any unnecessary stress. In the light of the results of baseline C-peptide concentrations it is doubtful that oral insulin had an effect on those of stimulated C-peptide. Older patients also did not benefit from the addition of oral insulin: thus, this antigen-based therapy, which is supposed to induce tolerance to a key antigen (e.g. insulin) in Type I diabetes, seems to be ineffective (at least at the doses used in this trial) in protecting residual beta-cell function in patients with recently diagnosed disease.

There are a number of possibilities to explain these findings. One is that the oral insulin daily dose used in this trial (5 mg) was not sufficient. Several experimental data have indicated that the dose of antigen is a critical factor for tolerance induction in autoimmune diseases [13]. A similar trial in France, in which two doses of oral insulin (2.5 mg and 7.5 mg) were used and preliminary data presented in abstract form did not show any effect [14]. As the addition of oral insulin does not influence metabolic control, higher doses of oral insulin should possibly be tested. The use of an adjuvant carrier to increase the tolerogen capacity of insulin is also worth consideration for tolerance induction, with implications for clinical use [15].

Another possibility is that at the time of clinical diagnosis of Type I diabetes residual beta-cell mass is so small that the efficacy of this treatment cannot be de-

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tected. This might be different in pre-Type I diabetes, in which beta-cell mass is almost unaffected and the spreading of the autoimmune response to a number of other antigens, which generally amplifies the rate of beta-cell destruction, is still limited. In such a case tolerance induction might still be possible with insulin. It is, however, of concern that in our trial treatment with oral insulin seemed to accelerate the decline of beta-cell function, at least in the very young subjects, because insulin is considered to be the major target of the autoimmune attack against beta cells, especially in young patients. In other autoimmune condition(s) the addition of oral antigens has induced an accelerating effect on disease progression [16]. A reasonable concern is that if oral insulin has no or negative effects on the natural course of beta-cell destruction in the first year after diagnosis it might have similar effects when given before the onset of overt hyperglycaemia. The trend towards lower IA responses observed in patients treated with oral insulin might reflect a modulation of the response induced against exogenous insulin. The reduction was, however, not statistically significant, so it is difficult to draw any definitive conclusion on the effect of oral insulin given at disease onset in terms of modulation of antigen-specific immune reactivity.

All these concerns apply to the prevention trials designed to test whether intervention during the prodromal period of Type I diabetes can delay its clinical onset. Specifically, the objective of the DPT-1 is to determine whether antigen-based therapies (e.g. insulin) in non-diabetic relatives of patients with Type I diabetes can delay the development of overt clinical disease [1]. Based on the results of our trial attention should be paid to the subjects in the oral treatment group of DPT-1 trial to find out whether oral insulin affects C-peptide secretion or insulin antibody concentrations.

In conclusion, the addition of 5 mg daily of oral insulin to regular subcutaneous insulin therapy has no effect on residual beta-cell function in patients with recent-onset Type I diabetes and does not modify the humoral immune response against the hormone. These results have important implications for current thoughts in designing strategies for preventing Type I diabetes.

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EXHIBIT H

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Exhibit E

Pathways for self-tolerance and the treatment of autoimmune diseases

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Christopher C Goodnow

Antigen delivers both immunogenic and tolerogenic signals to lymphocytes. The outcome of antigen exposure represents a complex integration of the timing of antigen binding with signals from many other immunogenic and tolerogenic costimulatory pathways. A road map of these signalling pathways is only beginning to be charted, revealing the mechanism of action and limitations of current immunotherapeutic agents and the points of attack for new agents. Ciclosporin and tacrolimus interfere with tolerogenic signals from antigen in addition to blocking immunogenic signals, thus preventing active establishment of tolerance. Corticosteroids inhibit a key immunogenic pathway, NFκB, and more specific inhibitors of this pathway may allow tolerance to be actively established while immune responses are blocked. New experimental therapies aim to mimic tolerogenic antigen signals by chronically stimulating antigen receptors with antigen or antibodies to the receptor, or aim to block costimulatory pathways involving CD40 ligand, B7, or interleukin 2. Obtaining the desired response with these strategies is unpredictable because many of these signals have both tolerogenic and immunogenic roles. The cause of autoimmune diseases has been determined for several rare monogenic disorders, revealing inherited deficiencies in tolerogenic costimulatory pathways such as FAS. Common autoimmune disorders may have a biochemically related pathogenesis.

Self-tolerance is an essential feature of the immune system, and works to protect tissue antigens from becoming targets of damaging immune responses during clearance of infection. The immune system normally exhibits exquisite specificity in distinguishing infectious antigens from self antigens. Vigorous antibody or T-cell responses are mounted against infectious antigens, whereas self antigens generally elicit only transient or weak responses even when incorporated into an infectious particle.

Adaptive immune responses start with the binding of antigen to antigen receptors on rare lymphocytes. The number and activity of these cells is then greatly expanded by clonal proliferation and differentiation. The response of individual lymphocytes is governed, however, by opposing immunogenic and tolerogenic signals, and the latter normally prevail for lymphocytes that bind self antigens. Disturbance in the natural balance between immunogenic and tolerogenic signals due to genetic factors can give rise to autoimmune disease. Progress in delineating these opposing signals provides opportunities to correct the primary disorder in autoimmune patients.

Counterbalancing immunogenic and tolerogenic signals

Two basic types of extracellular stimuli control lymphocyte growth and development (figure 1). The first is antigen signalling, through clone-specific antigen receptors. The second is costimuli, which encompasses a number of signals, through receptors that are not antigen specific. Importantly, particular antigen or costimuli signals are rarely obligately immunogenic or tolerogenic. Their timing and the way they are integrated at key checkpoints in lymphocyte development determines how a lymphocyte responds. Strongly immunogenic costimuli can shift the balance to immunity in the face of strongly tolerogenic antigen signals, and strongly tolerogenic costimuli can over-ride strongly immunogenic antigen signals. Deciphering the molecular logic behind this signal integration is the central challenge facing clinical manipulation of tolerance and immunity.

Immunogenic and tolerogenic antigen signals

Antigens transmit signals to lymphocytes by binding to B-cell receptors (surface immunoglobulin on B cells), and to T-cell receptors (TCRs) on T cells. B-cell receptors and TCRs signal through a cascade of protein tyrosine kinases and protein-lipid phosphorylation. Antigen transmits immunogenic or tolerogenic signals to lymphocytes through these receptors. Continuous

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Figure 1: **Schematic diagram illustrating the balance of immunogenic and tolerogenic signals affecting lymphocyte responses to antigen**
LPS=lipopolysaccharide.

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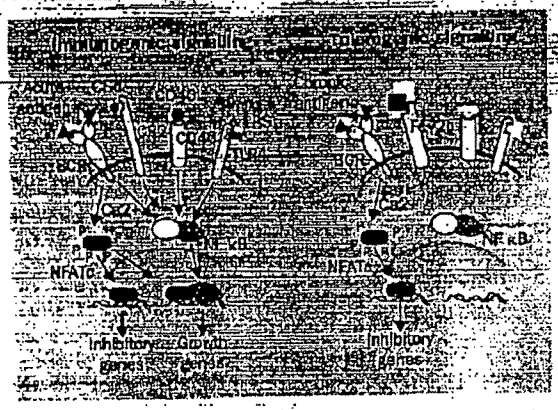

Figure 2: Biochemically distinct signals underpin immunogenic and tolerogenic responses to antigen in B lymphocytes

Immunogenic signalling occurs when antigen is encountered suddenly by mature B cells, and is augmented by co-clustering of complement C3d-receptor, CR2, and concurrent stimulation by CD40L from helper T cells or bacterial products such as lipopolysaccharide (LPS). One of the chief pathways activated by these signals is NFκB, a DNA binding protein family that moves to the nucleus once activated. In the nucleus, NFκB is pivotal to stimulating expression of many key B lymphocyte growth genes, promoting cell proliferation and antibody. The B-cell receptor also activates another DNA binding protein, NFATc, which moves to the nucleus after calcium-induced dephosphorylation and can work synergistically with NFκB. On its own, NFATc can activate inhibitory genes such as the inhibitory receptor CD72. Tolerogenic signalling occurs when antigen is encountered chronically, which results in inhibitory changes that diminish calcium signalling so that NFκB is no longer activated. Co-clustering of the receptor for IgG, FcγR2b, also inhibits immunogenic signalling to NFκB. Absence of costimuli such as CD40L or LPS is also critical to allow tolerogenic signalling to proceed in the absence of NFκB.

binding of antigen over several days, as is often the case for self antigens, usually transmits tolerogenic signals. By contrast, a sudden increase in receptor crosslinking, as occurs in most infections, tends to transmit immunogenic signals. Binding of antigen during immature lymphocyte formation in bone marrow or thymus, as occurs for many self antigens but few infectious antigens, tends to be tolerogenic.[1] Immunogenic signals are favoured when antigen is first encountered after lymphocytes have matured and reached the secondary lymphoid tissues, where infectious antigens tend to be trapped.

Immunogenic and tolerogenic antigens elicit different biochemical signals within lymphocytes[2] (figure 2). These biochemical differences provide opportunities to develop immunosuppressants that mirror these different signal patterns. In mature B lymphocytes, tolerogenic signalling by antigen elicits a smaller calcium response than immunogenic antigen. The calcium concentration achieved with tolerogenic signals is enough to activate the nuclear factor of activated T cells (NFATc) but insufficient to activate the nuclear factor kappa binding molecule (NFκB). NFATc and NFκB are DNA binding transcription factors that promote expression of different sets of genes. NFAT is essential for turning on lymphocyte inhibition as well as activatory genes, whereas NFκB is more purely immunogenic, because it is essential for inducing genes necessary for B and T cell proliferation and antibody production. As a result, a different pattern of gene expression is established by tolerogenic and immunogenic exposures to the same antigen.[3]

Deficiency of the NFκB transcription factor, c-rel, abolishes both T and B cells' immunogenic responses to antigen.[4] The inherited immunodeficiency syndrome, X-linked agammaglobulinaemia, is caused by defects in Bruton's tyrosine kinase (BTK), an intracellular enzyme that is essential for immunogenic signalling to NFκB by B-cell receptors.[5] Tolerogenic signalling to antigen remains intact or enhanced in BTK-defective B cells. This selective role in immunogenic signalling might explain the powerful suppression of systemic lupus in NZB/W mice when defects in BTK are introduced by breeding.[6] The selective role of the BTK/NFκB pathway in immunogenic signalling to antigen thus makes it an attractive target for new immunosuppressive drugs.

Immunogenic costimuli from microorganisms

Costimuli arise from many sources in the lymphocyte microenvironment. Perhaps the only purely immunogenic costimuli come from conserved components of infectious microorganisms. The lipopolysaccharide (LPS) moiety of bacterial cell walls and DNA rich in the dinucleotide, CpG from bacteria both activate the NFκB pathway in lymphocytes through surface receptors of the Toll-like receptor (TLR) family[7,8] (figure 2). These immunogenic costimuli also signal lymphocytes indirectly by activating antigen presenting cells—dendritic cells, macrophages, and B cells, to produce additional immunogenic costimuli such as the T cell activating cell surface protein B7 (CD80) and the inflammatory cytokine tumour necrosis factor alpha (TNFα). Bacterial adjuvants have been explored as experimental therapeutics to promote immunogenic responses to autoantigens on tumour cells but give rise to other undesirable inflammatory effects. Their effect may be more specifically emulated by activating dendritic cells bearing tumour antigens in vitro and giving these cells to the patient.

Costimuli from stressed and dying cells

Cell death through apoptosis occurs physiologically in healthy tissues without inflammation or immunogenicity. Engulfment of apoptotic cells by tissue macrophages, dendritic cells, or fibroblasts elicits signals through the phosphatidylserine receptor that promote synthesis of the tolerogenic cytokine, transforming growth factor beta (TGFβ; figure 3) and inhibit production of the immunogenic cytokine TNFα.[9] By contrast, pathological cell death by necrosis links antigens with immunogenic costimuli. Necrotic cells, and antigens released from necrotic or stressed cells complexed with the heat-shock proteins, Hsp96 and Hsp70, activate dendritic cells to express immunogenic costimuli including B7 and TNFα.[10–12] In patients and animals models with developing neoplasms, increased production of these immunogenic costimuli through cell dysplasia and necrosis may account for the frequent detection of subclinical autoantibodies and for the less frequent paraneoplastic autoimmune syndromes. The latter might simply reflect rare clinical manifestations of common autoimmune responses to dysplastic tumour cell autoantigens, as a result of chance reactivity of the autoantibodies with a vital cell receptor. Likewise, cell stress and dysfunction in specific organs, such as the pancreatic beta cell, may be an immunogenic costimulus for autoimmunity.

Dual role of the complement system

Activation of the serum complement system by foreign cells or particles produces powerfully immunogenic costimuli, partly by covalently tagging the infectious antigens with the complement cleavage product C3d.[13] C3d signals immunogenically to B lymphocytes, through the complement C3d receptors, CR1 and CR2 (CD21

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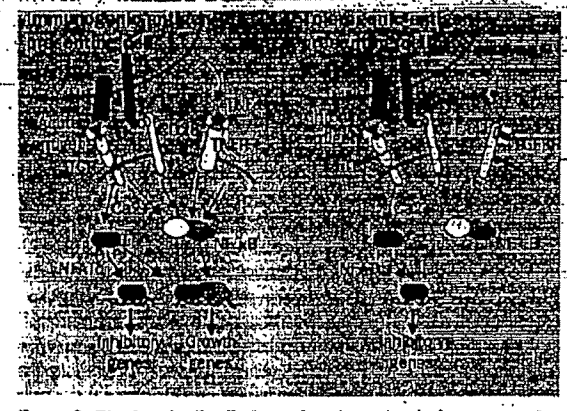

Figure 3: Biochemically distinct signals underpin immunogenic and tolerogenic responses to antigen in T lymphocytes

Immunogenic signalling occurs when antigen peptide and MHC complexes are encountered suddenly by mature T cells, and is augmented by concurrent stimulation by B7 molecules engaging CD28, or binding of TNFα. One of the chief pathways activated by these signals is NFκB, a DNA binding protein family that moves to the nucleus once activated. In the nucleus, NFκB is pivotal to stimulating expression of many key T lymphocyte growth genes, promoting cell proliferation and inflammatory cytokines. The TCR also activates another DNA binding protein, NFATc, which moves to the nucleus after calcium-induced dephosphorylation and can work synergistically with NFκB. On its own, NFATc can activate inhibitory genes such as the death receptor ligand, FasL. Tolerogenic signalling occurs when antigen is encountered chronically, which results in inhibitory changes that diminish calcium signalling so that NFκB is no longer activated. CD28 is downregulated and an inhibitory receptor for B7, CTLA4, is upregulated. Concurrent stimulation by TGFβ inhibits expression of lymphocyte growth genes.

and CD35), when a C3d-tagged antigen causes clustering of these receptors with the B-cell receptors (figure 2). Complement components C1, C2, C4, and the CR1/2 complement receptors are also important for delivering tolerogenic signals, since inherited deficiencies of these elements in human beings and mice are associated with susceptibility to autoimmune disease. C1q deficiency leads to an inability to clear apoptotic cells efficiently, and this may either diminish the tolerogenic signals elicited by physiological cell corpses or allow them to become immunogenic.[14]

Dual role of the B7 system

Cell surface proteins of the B7 family, displayed on antigen presenting cells such as macrophages, dendritic cells, and B lymphocytes, deliver immunogenic costimuli to T cells by signalling through the CD28 and inducible costimulator (ICOS) receptors[15,16] (figure 3). The B7.1 and B7.2 proteins are induced on antigen presenting cells by other immunogenic costimuli, such as LPS, necrotic cells, or immunogenic antigen receptor signals in B cells, creating a cascade of immunogenic signals. Immunosuppressive therapy aimed at blocking the immunogenic effects of B7.1 and B7.2, notably the recombinant protein antagonist CTLA4-Ig, has been shown to improve the symptoms of psoriasis.

B7/CD28 costimuli are tolerogenic in other contexts, notably in immature thymocytes where they enhance clonal deletion. The B7/CD28 pathway also promotes tolerance by signalling the formation of regulatory CD4+CD25+ T cells that may be required for tolerance to tissue antigens.[17] B7.1 and B7.2 proteins also transmit tolerogenic signals to T cells by engaging another receptor, CTLA4, that is present at very low levels in resting T cells and substantially increased by chronic antigen signals[18] (figure 3). The importance of CTLA4 as a brake to the system is shown by the lethal inflammatory and lymphoproliferative disorder that occurs in CTLA4-deficient mice, and by the augmented autoimmune responses to melanoma antigens that occur when CTLA4 is blocked with antibodies.

Dual role of TNFα family of proteins and receptors

Activation of T cells and other innate or adaptive immune cells elicits an important and growing class of immunogenic and tolerogenic costimuli related to the cytokine, TNFα. TNFα itself has a pleiotropic effect on immune responses and inflammatory cells.[19–21] In some contexts, TNFα promotes self-tolerance and CD8 T cell deletion, whereas in others TNFα promotes T cell activation and autoimmune disease. Inherited deficiencies in TNFα or its receptors in mice results in poor cytotoxic T-cell-mediated resistance to certain viruses and inability to form follicular dendritic cells needed for humoral immunity. Symptoms of rheumatoid arthritis improve after blocking TNFα with antibodies or recombinant protein antagonists, indicating that production of this cytokine by T cells in the synovium has a key inflammatory role.[22]

CD40-ligand (CD40L) and Fas-ligand are two proteins related to TNFα with essential regulatory functions. Both are membrane-bound proteins displayed on T cells following T-cell receptor signals. CD40L engages its receptor, CD40, on B cells and dendritic cells to activate immunogenic responses through the NFκB pathway.[23] The importance of CD40L as an immunogenic costimulus is shown in children and mice with inherited CD40L deficiency, the X-linked hyper-IgM syndrome, where there is an absence of IgG antibody responses and defective T-cell immunity. Experimental therapies based on blocking the immunogenic effects of CD40L on B cells and dendritic cells with antibodies showed spectacular promise in animal models, notably achieving long-term allograft tolerance in primates.[24] Clinical trials in human beings have been suspended, however, because of thromboembolic complications in a subset of participants.

CD40L also seems to have an important tolerogenic role, since CD40L-deficient children are also commonly affected by autoimmune disease. CD40L is needed as a tolerogenic signal for B cells to increase expression of Fas (CD95), the receptor for FAS-L.[25] FAS itself transmits a potent tolerogenic costimulus by triggering the death and deletion of self-reactive B and T lymphocytes. The importance of the FAS pathway is seen by the systemic Autoimmune Lymphoproliferative Syndrome (ALPS) in human beings and mice with inherited deficiencies in FAS-L, FAS, or the downstream protease Caspase-10.[25–26]

Inhibitory co-receptors

This is a rapidly growing class of receptors that transmit inhibitory or tolerogenic costimuli to lymphocytes functions by recruiting protein tyrosine or lipid phosphatases. The prototype for this family is the low affinity receptor for IgG, FcγR2b, on B cells.[27] Antigen and antibody complexes cause the antigen receptors to cluster with FcγR2b, preventing B cell activation by otherwise immunogenic antigens and, instead, triggering death and deletion of the B cells (figure 2). This mechanism is believed to explain the tolerogenic effect of anti-RhD prophylaxis, in which small amounts of IgG anti-RhD antibodies given to Rh-negative mothers prevent maternal antibody responses to fetal RhD antigen.

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Cytokines

Cytokines deliver both immunogenic and tolerogenic costimuli to lymphocytes. This balance is well illustrated by interleukins 2, 7, and 15.[21,22,60] All three signal T and B cells through multisubunit receptors that share a common gamma chain (γc). Inherited deficiency of the γc subunit accounts for X-linked Severe Combined Immunodeficiency, characterised by lack of naïve or memory T and B cells. IL-7 delivers essential costimuli through γc that promote naïve T and B cell formation in thymus and bone marrow and promote survival of naïve T cells in the lymph nodes. Similarly, growth and persistence of memory CD8 T cells is promoted primarily by IL-15. By contrast, the essential function of IL-2 in vivo is to deliver a tolerogenic costimulus, despite its original discovery in tissue culture as a so-called T-cell growth factor. Mice lacking IL-2 or the unique IL-2 receptor alpha subunit develop a severe T cell lymphoproliferative disease with numerous autoantibodies. IL-2 sensitises T cells to receive tolerogenic signals by the Fas receptor system, and may also be required to sustain a tolerogenic subset of CD4+CD25+ regulatory T cells.

Transforming growth factor beta (TGF-β) delivers an important tolerogenic signal to lymphocytes, and mice lacking this cytokine rapidly develop a lethal syndrome of lymphocyte hyperactivity and autoantibodies.[61] TGF-β inhibits the entry of lymphocytes into the cell cycle, and thus might establish a high tolerogenic threshold against which immunogenic signals from antigen and costimuli must work to initiate lymphocyte responses. The early response to immunogenic antigen is differentiated from tolerogenic antigen responses in part by rapid downregulation of inhibitory transcription factors in the former.[3] TGF-β seems likely to establish these inhibitory factors in quiescent and tolerised lymphocytes. TGF-β production by macrophages is induced by recognition and engulfment of cells that have died by physiological (non-inflammatory) apoptosis.[8] Macrophages, dendritic cells, and T cells making TGF-β seem to promote tolerance to self and foreign antigens in the eye, lung, and gut.[62,63] Linking antigen signals with TGF-β signals may be the basis for the experimental phenomenon of oral tolerance. Clinical trials are underway aimed at preventing type 1 diabetes or ameliorating multiple sclerosis by inducing oral tolerance to pro-insulin or myelin basic protein.

Integration of tolerogenic and immunogenic signals at different steps in the immune response

Integration and timing of antigen signals and costimuli occur at numerous checkpoints in lymphocyte development. These checkpoints are placed all along the developmental pathway, from those that delete newly formed B or T cells in the bone marrow and thymus through to those that abort the formation of terminally differentiated plasma cells or killer cells. Lymphocytes integrate antigen signals and costimuli very differently from one checkpoint to another, because expression of receptors and their intracellular response machinery change during development. The multiplicity of checkpoints exists presumably for two main reasons. First, no single mechanism can adequately ensure tolerance to all self antigens. Second, the existence of multiple mechanisms balances the need for tolerance against the need to use cells that crossreact between self and foreign antigens for rapid immunity against infection.[64]

Clonal deletion in central lymphoid tissues

In the bone marrow and thymus, antigens that cluster antigen receptors rapidly and avidly—which would be immunogenic for a mature lymphocyte—are almost exclusively tolerogens for newly-formed B and T cells.[25] The basis for the tolerogenic response of immature lymphocytes seems to be a result of many things: differences in the second messengers elicited by antigen receptors in immature cells, differences in the set of genes that can be triggered by second messengers, and presence of tolerogenic costimuli in the bone marrow and thymus microenvironments. Immature thymocytes are triggered to die even when antigen signals are linked with costimuli such as B7/CD28 that would be immunogenic to mature T cells. In immature B cells, continuous B-cell receptor engagement with strongly crosslinking self antigens, such as DNA or surface antigens on haematopoietic cells, delivers a tolerogenic signal that immediately arrests the cell's maturation and leads to clonal deletion within 1–3 days. Some of these arrested cells reach the spleen before dying, but they are extraordinarily refractory or anergic to immunogenic costimuli such as LPS and CD40. Particular combinations of immunogenic costimuli, such as CD40 and IL-4 from helper T cells, may be able to over-ride the powerfully tolerogenic signals from self antigen in these situations and break tolerance at this point.

Only a subset of self antigens are nevertheless present in sufficient quantity in the thymus and bone marrow to trigger clonal deletion. There is simply not enough antigen to signal deletion for most clones which recognise antigens present in trace quantities in the circulation or which are restricted to other tissues, such as the pancreatic islets, the brain, or the thyroid. Other mechanisms normally ensure tolerance to these antigens.

Clonal anergy

Self antigens that are present in lesser amounts in the bone marrow or thymus, or that cluster antigen receptors less avidly, can signal repeatedly to B and T cells without attaining the threshold needed to trigger arrest and death.[3,32] This constant "tickling" of antigen receptors by self antigens nevertheless transmits tolerogenic signals, activating feedback mechanisms that render the cell more refractory or anergic to immunogenic antigen signals. Anergy mediates B cell tolerance to self DNA and chromatin, and CD4 T cell tolerance to systemic and organ-specific antigens. In both B and T cells, anergy seems to involve a selective weakening of the connections between antigen receptors and the NFκB and JNK intracellular signalling pathways. Signalling through other intracellular pathways such as NFAT remains intact, so that a different set of tolerogenic genes is induced and immunogenic cell growth genes controlled by NFκB and JNK are not called into action. The weakening of connections to NFκB and JNK raises the threshold of immunogenic signalling needed to trip a self-reactive cell into multiplication. In B cells, a sudden burst of very avid antigen receptor clustering, or strong signals from LPS or CD40, allow sufficient signalling to the NFκB pathway to break anergy and drive the cell growth cycle.

Clonal deletion and regulation in peripheral lymphoid tissues

In addition to anergy, a series of peripheral deletion mechanisms catch self-reactive cells that reach the spleen, lymph nodes, and other organs.[25] These peripheral tolerance checkpoints act by shortening

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lymphocyte lifespan, inhibiting lymphocyte migration and recirculation, or causing rapid cell death in germinal centres or liver. These peripheral processes are for the most part poorly understood in biochemical terms, with the exception of the peripheral elimination of autoreactive B and T cells through the Fas cell death pathway.[31,32,36,37]

Pathogenesis of autoimmune diseases

How does autoimmune disease arise? Given the range of self-tolerance processes, and the difficulty eliciting or maintaining autoimmune responses by deliberate means (for example in medical and veterinary efforts to achieve immunological contraception or castration), it is reasonable to ask how tolerance to one or more self antigens fails in many people. The reason is as yet unknown, except for the rare patients with inherited monogenic disorders such as ALPS and X-linked hyper-IgM.

Most of the common autoimmune diseases also have an important inherited element, contributing as much as 50% of the population risk, and particular types of autoimmune diseases thus cluster in families. This inherited susceptibility is nevertheless complex involving combinations of many different gene alleles.[39] The strongest contributions are made by particular haplotypes of the major histocompatibility complex (MHC) and specific HLA alleles within the MHC, whose products present antigen peptides to T cells. Exactly how particular MHC alleles predispose to autoimmunity is not yet established, and one can hypothesise too much or too little presentation of particular antigens by products of susceptible HLA alleles. Correlations between autoimmune susceptibility and many other chromosomal regions have been found in human beings and mice, but the complexity of the inheritance pattern has made it challenging to identify the non-MHC susceptibility genes.

Four basic kinds of defect may potentially give rise to autoimmune disease, either alone or in combination. A central challenge for clinical immunology will be to define which of these faults actually applies for individual patients, since the nature of the deficit will determine the success or failure of emerging therapeutic strategies.

Insufficient tolerogenic signalling from antigen

In order for deletion, anergy, or regulation to be triggered by tolerogenic signalling through antigen receptors, a sufficient number of receptors must be engaged on self-reactive cells. Autoantigens that are only present in trace amounts in the lymphatic tissues will not achieve this signalling threshold on any but the very highest affinity clones. If the autoantigen is highly expressed in extralymphatic sites, as is the case for insulin, thyroglobulin, myelin proteins, skin basement proteins, and type 2 collagen, these concentrated depots of autoantigen might suddenly deliver an acute immunogenic stimulus to self-reactive cells that chance to migrate into these sites. This situation seems to be the case for B cells and some CD8 T cells.[35,40] For CD4 T cells recognising such antigens, there seems to be some autoantigen encountered in lymphatic sites that might induce anergy and regulatory cells.[35]

Several susceptibility genes for type 1 diabetes may act by further diminishing this already limiting pathway for tolerogenic autoantigen presentation. Diabetes-susceptible MHC Class II alleles in human beings and mice seem less efficient at presenting antigens, potentially explaining the heightened risk of autoimmunity in individuals who are homozygous for these alleles.[41] A variant allele of the insulin gene associated with type 1 diabetes susceptibility is expressed at lower levels in the thymus, potentially lessening presentation and education of regulatory T cells to this antigen.[42]

If the primary lesion in individuals susceptible to type 1 diabetes and other organ-specific diseases is simply one of inadequate tolerogenic signals from the target self antigens, then delivering more of these antigens in a tolerogenic form is a rational strategy. Obviously, this approach has the risk of inducing autoimmunity if the self antigen is delivered in an immunogenic form in some individuals, either due to the way the antigen is presented, to presence of immunogenic costimuli, or to presence of primed or memory lymphocytes that may be more refractory to tolerogenic signals. A better understanding of the molecular integration of tolerogenic and immunogenic signals may be critical to the success of specific vaccines against diabetes and other autoimmune diseases.

Insufficient tolerogenic signals from autoantigen might also explain shortcomings of the immunosuppressive drugs, ciclosporin and tacrolimus (FK506). These drugs block the calcium/calcineurin/NFAT signalling pathway. This pathway is continually activated by self antigen in anergic B and T cells, and is important for inducing tolerogenic costimuli on lymphocytes such as CD72 and FAS-L. Interference with these actively tolerogenic signals might explain the systemic autoimmune disorders that can occur after cessation of the drug, and might account for the inability to achieve long-term allograft acceptance with these agents.[1] The presence of circulating autoantibodies may compound autoimmunity in systemic lupus by blocking the presentation of tolerising autoantigens to B cells.[43]

Too much imunogenic signalling from antigen

Sudden presentation of viral or bacterial antigen in a highly crosslinked, immunogenic form, and associated with immunogenic costimuli produced by the infection, can provoke immune responses from T or B cells that crossreact with the microbial antigen and a self antigen. In animal models, this route can activate ignorant T and B cell clones that, through a combination of lower affinity receptors and limiting self-antigen presentation, had not received appreciable tolerogenic signals.[35,40,44,45] Moreover, if the immunogenic antigen stimulus is very strong, such as occurs with highly multimeric forms of antigen for B cells, the stimulus can overcome strongly tolerogenic antigen signals to break anergy[46,47] or over-ride clonal deletion.[48] Whereas a microbial trigger is postulated to be the cause of a number of common autoimmune diseases, perhaps the best established clinical example is the immunopathological damage of heart valves by antibodies that crossreact between valvular antigens and streptococcal M protein.

Interestingly, the self-reactive components of crossreactive responses are usually transient and lack memory in most experimental and practical situations in which tolerance is transiently broken by immunogenic delivery of self and foreign antigens. This phenomenon is a longstanding problem for medical and veterinary efforts to achieve immunocontraception and immunocastration, in which the autoantibody titres to pregnancy or sex hormones fall prematurely in the face of heightened titres to the foreign carrier proteins. Susceptibility to full-blown autoimmune disease might therefore require that a crossreactive trigger be coupled with deficits in the tolerogenic costimuli that normally create an inhibitory feedback on self antigen responses.

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Deficiency of tolerogenic costimuli

Many of the rare systemic autoimmune disorders that are inherited as monogenic traits in human beings and mice arise from deficiencies of tolerogenic costimuli. The clearest example is human autoimmune lymphoproliferative syndrome (ALPS), which results from partial or complete deficiency in signalling by the death receptor FAS.[12] Similarly, deficiency of CD40L in X-linked hyper-IgM syndrome is commonly accompanied by autoimmune disorders that might reflect the need for CD40L to induce Fas on self-reactive B cells.[13] The monogenic autoimmune disorders listed above are clinically distinct from the common forms of autoimmune disease, but they illustrate the essential and non-redundant role of tolerogenic costimuli as brakes on autoimmunity. Common autoimmune disorders probably arise from collections of more subtle gene variants that collectively diminish the same tolerogenic pathways. In support of this notion, the type 1 diabetes susceptibility gene in the NOD mouse, *Idd3*, seems to be a variant form of IL-2 that may reduce the in-vivo efficacy of this tolerogenic costimulus.[14]

Too much immunogenic costimuli

There are many artificially engineered animal models where overexpression of immunogenic costimuli predisposes to autoimmune disease. For example, mice that overexpress TNFα, B7.1, IL-2, or IL-4 on pancreatic islet β-cells are predisposed to type 1 diabetes.[15] Cell death by necrosis releases antigens complexed with immunogenic costimuli, notably the heat-shock proteins HSP70 and HSP96, and necrotic cells activate dendritic cells. An increase in these tolerogenic immunogenic costimuli might explain the immunogenicity of dysplastic tumours that are commonly manifested by the appearance of subclinical autoantibodies to tumour antigens and by paraneoplastic autoimmune syndromes. Along similar lines, the inability to clear dead cells or chromatin might provoke systemic lupus in people with complement C1q deficiency.[16]

Targets for current and future therapy of autoimmune disease

The unfolding of the human genome project will accelerate assembly of a molecular map of immunogenic and tolerogenic signalling pathways. Translating this knowledge into cures for common autoimmune diseases will involve researchers addressing two key challenges. First, we must develop ways to diagnose the underlying cause of autoimmune disease in individual patients. There is probably little to be gained by giving an exogenous source of tolerogenic costimuli such as TGF-β or Fas-ligand to patients with an underlying problem further downstream in the receptors or signal-transduction pathways for these molecules. Methods for obtaining a genetic fingerprint of thousands of immunologically relevant genes will soon become available, and these might provide a way to shortlist the likely pathogenic deficits in individuals. Confirmation will probably require diagnostic biomarkers or specific assays for discrete immunogenic or tolerogenic pathways that can be done on blood samples.

The second critical element is development of protein or small molecule therapeutics that target critical pathways, either augmenting tolerogenic pathways or blocking immunogenic ones. Some of the best current agents for treating systemic autoimmune diseases, such as glucocorticoids, choloroquine, and gold compounds, seem to work by blocking the immunogenic NFκB pathway.[17] Improvements on these agents depend on narrowing the action to specific subsets of lymphocytes and avoiding the undesirable metabolic effects of glucocorticoids. To cure fully developed autoimmunity, drug targets will need to come from understanding why memory T and B cells are more refractory to tolerogenic signals and why they are less dependent upon immunogenic costimuli.

Engineered proteins and antibodies aimed at blocking specific immunogenic costimuli upstream of NFκB, notably antibodies against TNFα,[18] CD40L,[19] and the blockers of B7 ligands of CD28, have shown great promise in mouse models and in clinical trials as agents to treat rheumatoid arthritis or establish transplantation tolerance. These strategies may be most effective in individuals with healthy tolerogenic signalling, such as patients undergoing organ transplantation, where the underlying defect is known to be an excess of immunogenic antigen and immunogenic costimuli. In this case, temporarily blocking the immunogenic signals selectively should allow tolerogenic antigen and costimuli to establish an active, reinforcing state of tolerance that persists when blocking therapy is stopped. However, if inherited deficits in tolerogenic signalling prevent restoration of tolerance during a brief window of blocking therapy, it will be necessary to continue the immunogenic blockers for long periods, even though there are many complications associated with long-term immunosuppression.

An attractive notion is the idea of so-called negative vaccines; vaccines that could deliver specific antigens in a way that augments tolerogenic rather than immunogenic signalling. In animal models, delivering low amounts of antigen by the mucosal route, either ingested or nasally, can act as a potent tolerogen. This process might perhaps work by linking the antigen with the tolerogenic costimulus, TGF-β, which features in mucosal immune responses.[20,21] The first clinical trial of oral tolerance was unsuccessful, pointing to the need to understand better the mechanisms involved and to develop ways to achieve more reliable linkage between tolerogenic antigen and suitable tolerogenic costimuli. Likewise, rational molecular strategies are needed to improve the success rate of empirical regimes for desensitising allergic reactions to pollens and venoms and to restore tolerance to blood products such as clotting factor VIII.

The one shining example of a successful tolerogenic vaccine is the prevention of erythroblastosis fetalis in Rh-antigen incompatible pregnancies by giving small amounts of anti-RhD antibody. The antibody converts an immunogen (fetal red cells) into a tolerogen by recruiting a tolerogenic costimulus, FcγR2b. The wide success and cost-benefit of this simple method is an example of how it should become possible to shift the balance back towards tolerance in an antigen specific way for many autoimmune diseases. The key lies in understanding the molecular interplay between immunogenic and tolerogenic pathways and having a way to forge the desired tolerogenic connections in specific lymphocyte clones.

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(54) Title: METHODS EMPLOYING AND COMPOSITIONS CONTAINING PLAQUE ASSOCIATED MOLECULES FOR PREVENTION AND TREATMENT OF ATHEROSCLEROSIS

(57) Abstract: Methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance and inhibiting inflammatory processes contributing to atheromatous vascular disease and sequalae are provided.

METHODS EMPLOYING AND COMPOSITIONS CONTAINING PLAQUE ASSOCIATED MOLECULES FOR PREVENTION AND TREATMENT OF ATHEROSCLEROSIS

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to atheromatous plaque associated molecules for prevention and treatment of atherosclerosis and related disease and, more particularly, to methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance and inhibiting inflammatory processes contributing to atheromatous vascular disease and sequalae.

Cardiovascular disease is a major health risk throughout the industrialized world. Atherosclerosis, the most prevalent of cardiovascular diseases, is the principal cause of heart attack, stroke, and gangrene of the extremities, and as such, the principal cause of death in the United States. Atherosclerosis is a complex disease involving many cell types and molecular factors (for a detailed review, see Ross, 1993, Nature 362: 801-809). The process, which occurs in response to insults to the endothelium and smooth muscle cells (SMCs) of the wall of the artery, consists of the formation of fibrofatty and fibrous lesions or plaques, preceded and accompanied by inflammation. The advanced lesions of atherosclerosis may occlude the artery concerned, and result from an excessive inflammatory-fibroproliferative response to numerous different forms of insult. For example, shear stresses are thought to be responsible for the frequent occurrence of atherosclerotic plaques in regions of the circulatory system where turbulent blood flow occurs, such as branch points and irregular structures.

The first observable event in the formation of an atherosclerotic plaque occurs when inflammatory cells such as monocyte-derived macrophages adhere to the vascular endothelial layer and transmigrate through to the sub-endothelial space. Elevated plasma LDL levels lead to lipid engorgement of the vessel walls, with adjacent endothelial cells producing oxidized low

density lipoprotein (LDL). In addition, lipoprotein entrapment by the extracellular matrix leads to progressive oxidation of LDL by lipoxygenases, reactive oxygen species, peroxynitrite and/or myeloperoxidase as well as other oxidizing compounds. These oxidized forms of LDLs are then taken up in large amounts by vascular cells through scavenger receptors expressed on their surfaces.

Lipid-filled monocytes and smooth-muscle derived cells are called foam cells, and are the major constituent of the fatty streak. Interactions between foam cells and the endothelial and smooth muscle cells surrounding them produce a state of chronic local inflammation which can eventually lead to activation of endothelial cells, increased macrophage apoptosis, smooth muscle cell proliferation and migration, and the formation of a fibrous plaque (Hajjar, DP and Haberland, ME, J.Biol Chem 1997 Sep 12; 272(37):22975-78). Such plaques occlude the blood vessels concerned and thus restrict the flow of blood, resulting in ischemia, a condition characterized by a lack of oxygen supply in tissues of organs due to inadequate perfusion. When the involved arteries block the blood flow to the heart, a person is afflicted with a 'heart attack'; when the brain arteries occlude, the person experiences a stroke. When arteries to the limbs narrow, the result is severe pain, decreased physical mobility and possibly the need for amputation.

Oxidized LDL has been implicated in the pathogenesis of atherosclerosis and atherothrombosis, by it's action on monocytes and smooth muscle cells, and by inducing endothelial cell apoptosis, impairing anticoagulant balance in the endothelium. Oxidized LDL also inhibits anti-atherogenic HDL-associated breakdown of oxidized phospholipids (Mertens, A and Holvoet, P, FASEB J 2001 Oct; 15(12):2073-84). This association is also supported by many studies demonstrating the presence of oxidized LDL in the plaques in various animal models of atherogenesis; the retardation of atherogenesis through inhibition of oxidation by pharmacological and/or genetic manipulations; and the promising results of some of the

interventionaltrials with anti-oxidant vitamins (see, for example, Witztum J and Steinberg, D, Trends Cardiovasc Med 2001 Apr-May;11(3-4):93-102 for a review of current literature). Indeed, oxidized LDL and malondialdehyde (MDA)-modified LDL have been recently proposed as accurate blood markers for 1st and 2nd stages of coronary artery disease (US Pat. Nos. 6,309,888 to Holvoet et al and 6,255,070 to Witztum, et al).

Reduction of LDL oxidation and activity has been the target of a number of suggested clinical applications for treatment and prevention of cardiovascular disease. Bucala, et al (US Pat. No. 5869534) discloses methods for the modulation of lipid peroxidation by reducing advanced glycosylation end product, lipid characteristic of age-, disease- and diabetes-related foam cell formation. Tang et al, at Incyte Pharmaceuticals, Inc. (US Pat. No. 5,945,308) have disclosed the identification and proposed clinical application of a Human Oxidized LDL Receptor in the treatment of cardiovascular and autoimmune diseases and cancer.

Another abundant atherogenesis-related plaque component is Beta $_2$-Glycoprotein I. Beta $_2$-Glycoprotein I (Beta2GPI) is a 50-kDa molecule that acts as an anticoagulant in *in-vitro* assays. Although the exact role of Beta2GPI in atherogenesis has yet to be elucidated, several relevant properties have been observed: 1) it is able to bind negatively charged phospholipids or phospholipid-expressing cells (apoptotic cells, activated platelets); 2) it is able to bind to modified cellular surfaces, enhancing their clearance by scavenging macrophages (Chonn A, et al J Biol Chem 1995; 270: 25845-49; and Thiagarajan P, et al Arterioscler Thromb Vasc Biol 1999; 19:2807-11); and 3) it is an important target for binding of autoimmune antiphospholipid antibodies (aPLs). Beta2GPI has to undergo structural alteration in order to be recognized by aPLs. This alteration may be initiated, for example, by binding to negatively charged phospholipids or high binding plates, but also *in vivo* by binding apoptotic cells that express phophatidylserine.

Recent studies investigating the importance of anti Beta2GPI antibodies in promoting a procoagulant state have focused on the effects of these antibodies on cellular and protein components of the coagulation system (endothelial cells, platelets and macrophages; tissue factor and coagulation factors). These studies indicate that anti Beta2GPI antibodies prevent the deactivation of platelets, sustaining their phagocytic clearance; interact with late endosomes of human endothelial cells; and suppress the inhibitory activity of the tissue factor pathway inhibitor. This association with coagulation events is consistent with Beta2GPIs proposed function in the prothrombotic antiphospholipid syndrome (APLS). Both US Pat Nos 5998223 and 5344758 (to Matsuura, et al and Krilis, et al, respectively) disclose the application of anti Beta2GPI antibodies, some to cryptic epitopes, for diagnostics in APLS and SLE. However, no therapeutic applications are disclosed by the authors.

A third important plaque-related component associated with atherogenesis is the 60/65 kDa heat shock protein (HSP). This mitochondrial protein is a member of the HSP family, which constitutes nearly 24 proteins displaying high degree of sequence homologies between different species. These proteins, as their name implies, are expressed in response to stresses including exposure to free radicals, heat, mechanical shear stress, infections and cytokines, and protect against unfolding and denaturation of cellular proteins. This has led to their designation as molecular 'chaperones'. However, HSP function may have undesired consequences, since over expression of HSPs may, under certain conditions promote an autoimmune reaction with resultant tissue damage. The mechanisms responsible for the HSP immune mediated damage are as yet unclear: it is presumed that cryptic, "non-self" neo-epitopes are exposed following their upregulation. Alternatively, it was suggested that cross-reaction exists between self-HSP and 'foreign' HSP epitopes introduced following infections which may trigger a pathological, autoimmune response against native HSP. Support for the involvement of HSP in autoimmunity is provided by studies documenting enhanced autoantibody as

well as cellular response to HSP 60/65 in several autoimmune diseases (Schoenfeld, Y et al Autoimmunity 2000 Sep; 15(2):199-202; US Pat No. 6130059 to Covacci, et al; and Gromadza G, et al Cerebrovascul Dis 2001, Oct; 12(3):235-39).

The link between HSP 65 and atherosclerosis was initially recognized by George Wick's group, who found that normocholesterolemic rabbits immunized with different antigens developed atherosclerosis, provided the preparation used for immunization contained complete Freund's adjuvant (CFA)(Xu, Q, et al Arterioscler Thromb 1992;12:789-99). Since the major constituent of CFA is heat killed mycobacterium tuberculosis, the principal component of which is the HSP-65, they reasoned that the immune response towards this component led to the development of atherosclerosis. This was confirmed when these authors demonstrated that immunization of animals with HSP 65 produced pronounced atherosclerosis, and that T cells from experimentally atherosclerotic rabbits overexpressed HSP-65, indicating a localized immune reaction restricted to the stressed arterial vessel. The importance of endogenous HSP-65 in atherogenesis was further demonstrated by the acceleration of fatty streak formation following HSP-65 (or Mycobacterium tuberculosis) immunization of naïve mice (George J, et al Arterioscler Thromb Vasc Biol 1999; 19:505-10;).

Involvement of humoral immune mechanisms in response to HSP-65 were observed in atherosclerosis: a marked correlation has been found between high levels of anti-HSP65 antibodies and the extent of sonographically estimated carotid narrowing in a screen of healthy individuals (Xu Q. et al Lancet 1993; 341: 255-9; Xu Q. et al Circulation 1999; 100(11):1169-74). In addition, in-vitro experiments with cultured endothelial cells have demonstrated the concentration and time dependent induction of endothelial cell adhesion to monocytes and granulocytes following incubation with HSP65.

The association of HSP 65 with atherogenesis has led to a number of proposed therapeutic applications. Observing that immune reactivity to HSP 65 correlated with both microbial (e.g. *H. pylori*) infection and atherosclerosis, Bernie et al (Eur Heart J 1998; 19:366-7) proposed antibiotic therapy for reduction of infection and anti- HSP antibodies. Similarly, Covacci, et al (US Pat No. 6130059) disclosed the use of *H. pylori* HSPs, and related peptides, for diagnostic and therapeutic applications in atherosclerosis.

Atherosclerosis and autoimmune disease

Because of the presumed role of the excessive inflammatory-fibroproliferative response in atherosclerosis and ischemia, a growing number of researchers have attempted to define an autoimmune component of vascular injury. In autoimmune diseases the immune system recognizes and attacks normally non-antigenic body components (autoantigens), in addition to attacking invading foreign antigens. The autoimmune diseases are classified as auto- (or self-) antibody mediated or cell mediated diseases. Typical autoantibody mediated autoimmune diseases are myasthenia gravis and idiopathic thrombocytopenic purpura (ITP), while typical cell mediated diseases are Hashimoto's thyroiditis and type I (Juvenile) Diabetes.

The recognition that immune mediated processes prevail within atherosclerotic lesions stems from the consistent observation of lymphocytes and macrophages in the earliest stages, namely the fatty streaks. These lymphocytes, which include a predominant population of CD4+ cells (the remainder being CD8+ cells) were found to be more abundant than macrophages in early lesions, as compared with the more advanced lesions, in which this ratio tends to reverse. These findings posed questions as to whether they reflect a primary immune sensitization to a possible antigen or alternatively, result from previously induced local tissue damage. Regardless of the factors responsible for the recruitment of these inflammatory cells to the early plaque, they seem to exhibit an activated state manifested by concomitant expression of MHC class II HLA-DR and interleukin (IL) receptor as well as

leukocyte common antigen (CD45R0) and the very late antigen 1 (VLA-1) integrin. Thus, the inflammatory reaction of the early stages of the atherosclerotic lesion may be either the primary initiating event leading to the production of various cytokines by the local cells (i.e endothelial cells, macrophages, smooth muscle cells and inflammatory cells), or one form of the immune system's response to the hazardous process. Some of the cytokines which have been shown to be upregulated by the resident cells include TNF-α, IL-1, IL-2, IL-6, IL-8, IFN-γ and monocyte chemoattractant peptide-1 (MCP-1). Platelet derived growth factor (PDGF) and insulin-like growth factor (ILGF) which are expressed by all cellular constituents within atherosclerotic plaques have also been shown to be overexpressed, thus possibly intensifying the preexisting inflammatory reaction by a co-stimulatory support in the form of a mitogenic and chemotactic factor. Recently, Uyemura et al. (J Clin Invest 1996; 97: 2130-2138) have elucidated type 1 T-cell cytokine pattern in human atherosclerotic lesions exemplified by a strong expression of IFN-γ but not IL-4 mRNA in comparison with normal arteries. Furthermore, IL-12 - a T-cell growth factor produced primarily by activated monocytes and a selective inducer of Th1 cytokine pattern, was found to be overexpressed within lesions as manifested by the abundance of its major heterodimer form p70 and p40 (its dominant inducible protein) mRNA.

Similar to the strong evidence for the dominance of the cellular immune system within the atherosclerotic plaque, there is also ample data supporting the involvement of the local humoral immune system. Thus, deposition of immunoglobulins and complement components has been shown in the plaques in addition to the enhanced expression of the C3b and C3Bi receptors in resident macrophages.

Valuable clues with regard to the contribution of immune mediated inflammation to the progression of atherosclerosis come from animal models. Immunocompromised mice (class I MHC deficient) tend to develop

accelerated atherosclerosis as compared with immune competent mice. Additionally, treatment of C57BL/6 mice (Emeson EE and Shen ML Am J Pathol 1993; 142: 1906-1915) and New-Zealand White rabbits (Roselaar SE , et al J Clin Invest 1995; 96: 1389-1394) with cyclosporin A, a potent suppressor of IL-2 transcription resulted in a significantly enhanced atherosclerosis under "normal" lipoprotein "burden". These latter studies may provide insight into the possible roles of the immune system in counteracting the self-perpetuating inflammatory process within the atherosclerotic plaque.

Atherosclerosis is not a classical autoimmune disease, although some of its manifestations such as the production of the plaque that obstructs the vasculature may be related to aberrant immune responsiveness. In classical autoimmune disease, one can often define very clearly the sensitizing autoantigen attacked by the immune system and the component(s) of the immune system which recognize the autoantigen (humoral, i.e. autoantibody or cellular, i.e. lymphocytes). Above all, one can show that by passive transfer of these components of the immune system the disease can be induced in healthy animals, or in the case of humans the disease may be transferred from a sick pregnant mother to her offspring. Many of the above are not prevailing in atherosclerosis. In addition, the disease definitely has common risk factors such as hypertension, diabetes, lack of physical activity, smoking and others, the disease affects elderly people and has a different genetic preponderance than in classical autoimmune diseases.

Treatment of inflammatory disease may be directed towards suppression or reversal of general and/or disease-specific immune reactivity. Thus Aiello, for example (US Pat. Nos. 6,034,102 and 6,114,395) discloses the use of estrogen-like compounds for treatment and prevention of atherosclerosis and atherosclerotic lesion progression by inhibition of inflammatory cell recruitment. Similarly, Medford et al (US Pat. No. 5,846,959) disclose methods for the prevention of formation of oxidized PUFA, for treatment of cardiovascular and non-cardiovascular inflammatory diseases mediated by the

cellular adhesion molecule VCAM-1. Furthermore, Falb (US Pat. No. 6,156,500) designates a number of cell signaling and adhesion molecules abundant in atherosclerotic plaque and disease as potential targets of anti-inflammatory therapies.

Since oxidized LDL, Beta2GPI and HSP 65 have been clearly implicated in the pathogenesis of atherosclerosis (see above), the contribution of these prominent plaque components to autoimmunity in atheromatous disease processes has been investigated.

Immune responsiveness to plaque associated molecules

It is known that Ox LDL is chemotactic for T-cells and monocytes. Ox LDL and its byproducts are also known to induce the expression of factors such as monocyte chemotactic factor 1, secretion of colony stimulating factor and platelet activating properties, all of which are potent growth stimulants. The active involvement of the cellular immune response in atherosclerosis has recently been substantiated (Stemme S, et al, Proc Natl Acad Sci USA 1995; 92: 3893-97), who isolated CD4+ within plaques clones responding to Ox LDL as stimuli. The clones corresponding to Ox LDL (4 out of 27) produced principally interferon-γ rather than IL-4. It remains to be seen whether the above T-cell clones represent mere contact with the cellular immune system with the inciting strong immunogen (Ox LDL) or that this reaction provides means of combating the apparently indolent atherosclerotic process.

The data regarding the involvement of the humoral mechanisms and their meaning are much more controversial. One recent study reported increased levels of antibodies against MDA-LDL, a metabolite of LDL oxidation, in women suffering from heart disease and/or diabetes (Dotevall, et al., Clin Sci 2001 Nov; 101(5): 523-31). Other investigators have demonstrated antibodies recognizing multiple epitopes on the oxidized LDL, representing immune reactivity to the lipid and apolipoprotein components (Steinerova A, et al., Physiol Res 2001;50(2): 131-41) in atherosclerosis and other diseases, such as diabetes, renovascular syndrome, uremia, rheumatic

fever and lupus erythematosus. Several reports have associated increased levels of antibodies to Ox LDL with the progression of atherosclerosis (expressed by the degree of carotid stenosis, severity of peripheral vascular disease etc.). Most recently, Sherer et al (Cardiology 2001;95(1):20-4) demonstrated elevated levels of antibodies to cardiolipin, β-2GPI and oxLDL, but not phosphatidylcholine or endothelial cells in coronary heart disease. Thus, there seems to be a consensus as to the presence of anti-plaque-component antibodies in the form of immune complexes within atherosclerotic plaque.

Antibodies to Ox LDL have been implicated in both normal and pathological lipoprotein metabolism. Thus, it is known that immune complexes of Ox LDL and its corresponding antibodies are taken up more efficiently by macrophages in suspension as compared with Ox LDL. No conclusions can be drawn from this consistent finding on the pathogenesis of atherosclerosis since the question of whether the accelerated uptake of Ox LDL by the macrophages is beneficial or deleterious has not yet been resolved.

Important data as to the significance of the humoral immune system in atherogenesis comes from animal models: hyperimmunization of LDL-receptor deficient rabbits with homologous oxidized LDL, resulted in the production of high levels of anti-Ox LDL antibodies and was associated with a significant reduction in the extent of atherosclerotic lesions. Likewise, a decrease in plaque formation followed the immunization of rabbits with cholesterol rich liposomes and stimulation of production of anti-cholesterol antibodies; however, this effect was accompanied by a undesirable 35% reduction in very low density lipoprotein cholesterol levels.

Regarding the immunogenicity of Beta2GPI, it has been shown that Beta2GPI serves as a target antigen for an immune-mediated attack, influencing the progression of atherosclerosis in humans and mice. George J et al. immunized LDL-receptor deficient mice with Beta2GPI, producing a pronounced humoral immune response to human Beta2GPI, and larger early

atherosclerotic lesions in comparison with controls (George J, et al Circulation 1998; 15:1108-15). Afek A, et al obtained similar results in atherosclerosis-prone apolipoprotein-E-knockout mice immunized once with human Beta2GPI and fed a high fat diet for 5 weeks (Afek A et al. Pathobiology 1999;67:19-25).

Further, although immune reactivity to Beta2GPI in humans with the prothrombotic antiphospholipid syndrome has traditionally been attributed to the presence of autoantibodies to Beta2GPI, recent observations have indicated the importance of a cellular immune response to Beta2GPI. T-cells reactive with Beta2GPI have been demonstrated in the peripheral blood of patients with antiphospholipid syndrome. These T cells displayed a T-helper-1 phenotype (secreting the proinflammatory (and proatherogenic) cytokine interferon-) and were also capable of inducing tissue factor production (Visvanathan S, and McNiel HP. J Immunolog 1999; 162:6919-25). Taken together, the abundant data gathered to date regarding anti Beta2GPI (for review see Roubey RA, Curr Opinion Rheumatol 2000; 12:374-378), indicates that the immune response to this plaque related antigen may play a significant role in influencing the size and composition of atherosclerotic plaque.

Finally, there exists a significant dependency in the antigenicity, and pathogenicity of oxidized phospholipids and Beta2GPI. As mentioned above, some of the autoimmune epitopes associated with minimally modified LDL and Beta2GPI are cryptic. Kyobashi, et al (J Lipid Res 2001; 42:697-709), and Koike, et al (Ann Med 2000; 32:Suppl I 27-31) have identified a macrophage-activating oxLDL specific ligand present only with Beta2GPI- OxLDL complex formation. This ligand was recognized by APLS-specific autoantibodies. Thus, both the pathogenic role of oxidized LDL and other plaque components, and their importance as autoantigens in atherosclerosis, as well as other diseases, have been extensively demonstrated in laboratory and clinical studies.

Mucosal Tolerance in Treatment of Autoimmune Disease

Recently, new methods and pharmaceutical formulations have been found that are useful for treating autoimmune diseases (and related T-cell mediated inflammatory disorders such as allograft rejection and retroviral-associated neurological disease). These treatments induce tolerance, orally or mucosally, e.g. by inhalation, using as tolerizers autoantigens, bystander antigens, or disease-suppressive fragments or analogs of autoantigens or bystander antigens. Such treatments are described, for example, in US Pat. No. 5,935,577 to Weiner et al. Autoantigens and bystander antigens are defined below (for a general review of mucosal tolerance see Nagler-Anderson, C., Crit Rev Immunol 2000;20(2):103-20). Intravenous administration of autoantigens (and fragments thereof containing immunodominant epitopic regions of their molecules) has been found to induce immune suppression through a mechanism called clonal anergy. Clonal anergy causes deactivation of only immune attack T-cells specific to a particular antigen, the result being a significant reduction in the immune response to this antigen. Thus, the autoimmune response-promoting T-cells specific to an autoantigen, once anergized, no longer proliferate in response to that antigen. This reduction in proliferation also reduces the immune reactions responsible for autoimmune disease symptoms (such as neural tissue damage that is observed in multiple sclerosis; MS). There is also evidence that oral administration of autoantigens (or immunodominant fragments) in a single dose and in substantially larger amounts than those that trigger "active suppression" may also induce tolerance through anergy (or clonal deletion).

A method of treatment has also been disclosed that proceeds by active suppression. Active suppression functions via a different mechanism from that of clonal anergy. This method, discussed extensively in PCT Application PCT/US93/01705, involves oral or mucosal administration of antigens specific to the tissue under autoimmune attack. These are called "bystander antigens". This treatment causes regulatory (suppressor) T-cells to be induced in the gut-

associated lymphoid tissue (GALT), or bronchial associated lymphoid tissue (BALT), or most generally, mucosa associated lymphoid tissue (MALT) (MALT includes GALT and BALT). These regulatory cells are released in the blood or lymphatic tissue and then migrate to the organ or tissue afflicted by the autoimmune disease and suppress autoimmune attack of the afflicted organ or tissue. The T-cells elicited by the bystander antigen (which recognize at least one antigenic determinant of the bystander antigen used to elicit them) are targeted to the locus of autoimmune attack where they mediate the local release of certain immunomodulatory factors and cytokines, such as transforming growth factor beta (TGF beta), interleukin-4 (IL-4), and/or interleukin-10 (IL-10). Of these, TGF-beta is an antigen-nonspecific immunosuppressive factor in that it suppresses immune attack regardless of the antigen that triggers the attack. (However, because oral or mucosal tolerization with a bystander antigen only causes the release of TGF-beta in the vicinity of autoimmune attack, no systemic immunosuppression ensues.) IL-4 and IL-10 are also antigen-nonspecific immunoregulatory cytokines. IL-4 in particular enhances (T helper 2) Th_2 response, i.e., acts on T-cell precursors and causes them to differentiate preferentially into Th_2 cells at the expense of Th_1 responses. IL-4 also indirectly inhibits Th_1 exacerbation. IL-10 is a direct inhibitor of Th_1 responses. After orally tolerizing mammals afflicted with autoimmune disease conditions with bystander antigens, increased levels of TGF-beta, IL-4 and IL-10 are observed at the locus of autoimmune attack (Chen, Y. et al., Science, 265:1237-1240, 1994). The bystander suppression mechanism has been confirmed by von Herreth et al., (J. Clin. Invest., 96:1324-1331, September 1996).

More recently, oral tolerance has been effectively applied in treatment of animal models of inflammatory bowel disease by feeding probiotic bacteria (Dunne, C, et al., Antonie Van Leeuwenhoek 1999 Jul-Nov;76(1-4):279-92), autoimmune glomerulonephritis by feeding glomerular basement membrane (Reynolds, J. et al., J Am Soc Nephrol 2001 Jan;12(1): 61-70) experimental

allergic encephalomyelitis (EAE, which is the equivalent of multiple sclerosis or MS), by feeding myelin basic protein (MBP), adjuvant arthritis and collagen arthritis, by feeding a subject with collagen and HSP-65, respectively. A Boston based company called Autoimmune has carried out several human experiments for preventing diabetes, multiple sclerosis, rheumatoid arthritis and uveitis. The results of the clinical trials have been less impressive than the animal experiments, however there has been some success with the prevention of arthritis.

Oral tolerance to autoantigens found in atherosclerotic plaque lesions has also been investigated. Study of the epitopes recognized by T-cells and Ig titers in clinical and experimental models of atherosclerosis indicated three candidate antigens for suppression of inflammation in atheromatous lesions: oxidized LDL, the stress-related heat shock protein HSP 65 and the cardiolipin binding protein beta 2GP1. US Patent Application 09/806,400 to Shoenfeld et al (filed Sept 30, 1999), which is incorporated herein in its entirety, discloses the reduction by approximately 30% of atherogenesis in the arteries of genetically susceptible LDL receptor deficient mice (LDL-RD) fed oxidized human LDL. Although significant inhibition of atherogenesis was achieved, presumably via oral tolerance, no identification of specific lipid antigens or immunogenic LDL components was made. Another obstacle encountered was the inherent instability of the orally fed antigen in vivo, due to digestive breakdown, and uptake of oxidized LDL by the liver and cellular immune mechanisms. It is plausible that a mucosal route of administration other than feeding (oral) would have provided tolerance of greater efficiency.

The induction of immune tolerance and subsequent prevention or inhibition of autoimmune inflammatory processes has been demonstrated using exposure to suppressive antigens via mucosal sites other than the gut. The membranous tissue around the eyes, the middle ear, the respiratory and other mucosa, and especially the mucosa of the nasal cavity, like the gut, are exposed to many invading as well as self- antigens and possess mechanisms

for immune reactivity. Thus, Rossi, et al (Scand J Immunol 1999 Aug;50(2):177-82) found that nasal administration of gliadin was as effective as intravenous administration in downregulating the immune response to the antigen in a mouse model of celiac disease. Similarly, nasal exposure to acetylcholine receptor antigen was more effective than oral exposure in delaying and reducing muscle weakness and specific lymphocyte proliferation in a mouse model of myasthenia gravis (Shi, FD. et al, J Immunol 1999 May 15; 162 (10): 5757-63). Therefore, immunogenic compounds intended for mucosal as well as intravenous or intraperitoneal administration should be adaptable to nasal and other membranous routes of administration.

Thus, there is clearly a need for novel methods of employing, and compositions of plaque associated molecules capable of superior tolerizing immunogenicity in mucosal, especially nasal, administration.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a pharmaceutical composition for prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, comprising, as an active ingredient, a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule, or pharmaceutical salts thereof, the composition further comprising a pharmaceutically acceptable carrier, the pharmaceutical composition being designed for mucosal administration.

According to an additional aspect of the present invention there is provided an assay for determining the effect of mucosal administration of plaque components on atherosclerosis-related disease or condition, the assay effected by mucosally administering to a subject having an atherosclerosis-related disease or condition at least an antigenic portion of at least one plaque associated molecule and assessing at least one indicator of atherogenesis in the

subject to thereby determine the effect of mucosal administration of the at least an antigenic portion of the at least one plaque associated molecule on the atherosclerosis-related disease or condition.

According to yet another aspect of the present invention there is provided a method of prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, the method comprising mucosally administering a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule to the subject.

According to further features in preferred embodiments of the invention described below, the plaque associated molecule is selected from the group consisting of oxidized LDL, Beta2GPI, HSP and derivatives thereof.

According to still further features in preferred embodiments of the invention described below, the antigenic portion of at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.

According to further features in preferred embodiments of the invention described below, the pharmaceutical composition is designed for nasal, respiratory, otic and/or conjuctival administration.

According to yet further features in preferred embodiments of the invention described below the at least an antigenic portion of the at least one plaque associated molecule is selected so as to reduce immune reactivity to plaque components in said subject.

According to further features in preferred embodiments of the invention described below, the pharmaceutical composition is packaged and identified for use in the prevention and/or treatment of at least one disorder selected from the group consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

According to further features in preferred embodiments of the invention described below the pharmaceutical composition further comprises a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a novel method of inducing superior immune tolerance by mucosal administration of plaque associated molecules, thereby inhibiting atheroslerosis and other plaque related diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 illustrates inhibition of early atherogenesis in apo-E deficient mice by nasal tolerance induced by administration of low doses of plaque associated molecules. 9-13 week old apo-E deficient mice were exposed intranasally, with mild sedation, to 3 doses of 10 μg/mouse each HSP 65 (HSP-65)(n=12), human oxidized LDL (H-oxLDL)(n=14), human Beta2GPI (B2gpi)(n=13), bovine serum albumin (BSA) or sham exposure to saline

(PBS)(n=12). All mice received the atherogenic "Western" diet following last exposure. Atherogenesis is expressed as the area of atheromatous lesions in the aortic sinus 5 weeks following the 3rd exposure.

FIG. 2 illustrates superior inhibition of early atherogenesis in apo-E deficient mice by mucosal tolerance induced by intranasal exposure to exceedingly low doses of HSP 65. Nasal tolerance was induced in 12-16 week old apo-E deficient mice by intranasal administration of 3 doses of 1μg/mouse HSP65 (HSP-65 low)(n=16) or 10μg/mouse HSP65 (HSP-65 high)(n=14) every other day for 5 days. Control mice were exposed intranasally to an identical volume (10μl) of bovine serum albumin, 10μg/mouse (BSA)(n=14), or sham exposure to PBS (PBS)(n=14). All mice received the atherogenic "Western" diet following last exposure. Atherogenesis is expressed as the area of atheromatous lesions in the aortic sinus 5 weeks after the last nasal exposure.

FIG. 3 illustrates superior suppression of immune reactivity to atheroslerotic plaque antigens induced by nasal exposure to human Beta2GPI. 5 week old male apo-E deficient mice were exposed intranasally to 10μg/mouse human Beta2GPI (H-b2-nt)(n=3); or alternately fed, by gavage, with 100μg/mouse human Beta2GPI (H-b2-ot)(n=3) in 0.2 ml PBS; or fed PBS alone (PBS)(n=3) every other day for 5 days. One week following the last feeding the mice were sensitized with a single subcutaneous injection of 10μg/mouse human Beta2GPI in 0.1 ml volume. Ten days later T-cells from inguinal lymph node were prepared as described in Materials and Methods section that follows, and exposed to the sensitizing human Beta2GPI antigen for in-vitro assessment of proliferation. Proliferation, indicating immune reactivity, is expressed as the ratio between incorporation of labeled thymidine into the T-cell's DNA in the presence and absence of human Beta2GPI antigen (stimulation index, S.I.).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods and compositions employing plaque associated molecules effective in inducing mucosal tolerance to atheroma related antigens, thus inhibiting inflammatory processes contributing to atheromatous vascular disease and sequalae.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Experimental and clinical evidence indicates a causative role for plaque associated antigens in the etiology of the excessive inflammatory response in atherosclerosis. Both cellular and humoral immune reactivity to the plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 have been demonstrated, suggesting an important anti-oxidized LDL auto-immune component in atherogenesis. Thus, oxidized LDL, Beta2GPI and HSP 65, and components thereof, have been the targets of numerous therapies for prevention and treatment of heart disease, cerebral-vascular disease and peripheral vascular disease.

Prior art teaches the application of plaque associated antigens for detection and diagnosis of atherosclerosis and other plaque- and thrombosis related conditions. For example, Holvoet (US Pat No. 6,309,888) teaches the use of stage specific plaque associated antigens oxLDL and MDA-LDL for screening for Coronary Artery disease. Similarly, others (US Pat Nos. 5,998,223 and 5,344,758 to Matsuura, et al and Krilis, et al, respectively) have disclosed the use of anti beta-2GPI antibodies to screen for serum indicators of

APLS, SLE and atherosclerosis. The abovementioned disclosures propose diagnostic applications alone, and fail to recognize the therapeutic potential of these plaque associated molecules.

Although the role of immune response in the etiology and progression of atherosclerosis and other plaque related diseases remains controversial (see Meir, K, et al, International Atherosclerosis Soc. 2001 Commentary), many immune-based therapies have been proposed for atherosclerosis. General methods of reducing immune response in inflammatory and hyperreactive conditions are taught in, for example US Patent Nos 6,277,969; 5,698,195 and 5,656,272 to Le at al, and 6,224,902 to Alving, et al, International Patent Application Nos. 001 001 2514 to Shurkovitz et al and 20010051156 A1 to Zeng. However, the proposed reduction or removal of mediators of immune reactivity, such as cytokines, tumor necrosis factor (TNF) and other pathogenic factors requires ongoing costly and potentially dangerous methods such as immunoadsorption of blood and prolonged anti-cytokine administration. Furthermore, no application to treatment of atherosclerosis or plaque-related disease is disclosed.

Specific immunotherapy with plaque associated antigens has also been proposed. Bumol, et al, Calenoff, et al and Takano, et al (US Pat Nos. 5,196,324; 6,025,477 and 5,110,738, respectively) disclose the use of crude, poorly defined fractionated plaque preparations for immunization, monoclonal Ab preparation, diagnosis and treatment of atherosclerosis. These antigens, protein and lipid fractions of atheromatous tissue, are poorly defined, impractical for therapeutic use, and potentially hazardous in prolonged treatment.

Prior art teaches immunotherapy directed against oxidized LDL for treatment and prevention of atherosclerosis. US Pat No. 6,225,070 to Witztum, et al discloses the use of mAb to oxidized LDL for inhibition of oxidized LDL binding to macrophages and foam cell formation. Similarly, McGregor, et al (International Patent Application EP1162458 A1) disclose

methods for specific modulation of oxidized LDL uptake and transport by macrophages. US Pat Nos 5,733,524 and 5,733,933 to Bucala, et al disclose the reduction of specific anti-oxidized LDL immune response by reduction of Advanced Glycosylation End product lipids (AGE-lipids). None of the proposed therapies teach active immunization against oxidized LDL, and require prolonged therapy regimens.

Zhou, et al (Arterioscler Thromb Vasc Biol, 2001;21:108) achieved a significant reduction in early plaque formation in mice following footpad immunization with homogenized plaque or homologous MDA LDL. Palinski et al (PNAS USA 1995;92:821-25) produced similar levels of protection in rabbits immunized with oxidized LDL. However, application of conventional immunization techniques to oxidized LDL is problematic, since the adjuvant preparations required for immunization and boosters have produced accelerated plaque formation in similar regimen of immunization. Furthermore, relatively high doses (100 µgram/ mouse/ injection) of plaque antigen were required for immunity. Mucosal administration and induction of tolerance were not mentioned.

Immune therapy with other plaque antigens has also been proposed. Recent animal and in-vitro studies with Beta2GPI (see George J, et al Rheum Dis Clin North Am 2001;27:603-10; Brey, et al Stroke 2001;32:1701-06; Kyobashi, et al J Lipid Res 2001;42:697-709; Koike T, et al Ann Med 2000;32,Suppl. I:27-31 and Cabral AR et al Am J Med 1996;101:472-81) have demonstrated the association of Beta2GPI with stroke, APLS, atherosclerosis and myocardial infarction. Although cryptic epitopes of the protein were clearly implicated in humoral and cellular immune response to oxidized LDL, none of the abovementioned studies demonstrated protective immunity with the protein. Similarly, studies with HSP 65 (Birnie DH Eur Heart J 1998;19:366-67; Xu Q, et al Circulation 1999;100:1169-74; and Gromadzka G, et al Cerebrovasc Dis 2001;12:235-39) have implicated this plaque

associated antigen in stroke and heart disease, suggesting that humoral immunity may be a triggering factor.

The complexity of plaque antigen immunity in atherosclerosis was demonstrated by Schoenfeld Y, et al (Autoimmunity 2000;15:199-202) who immunized LDL-receptor deficient (KO) mice with both HSP 65 and Beta2GPI protein antigens, producing strong cellular and humoral responses, and enhanced plaque formation. Similar increased atherogenesis was observed with passive transfer of plaque antigen activated lymphocytes. None of the above mentioned studies demonstrated inhibition of atherogenic processes by immune tolerance.

Suppression of immune response to autoantigens in atherosclerosis and related disease has been recently investigated. Victoria et al (US Pat. No. 6,207,160 and 5,844,409) discloses specific non-immunogenic Beta2GPI peptides lacking T cell epitopes for reducing antibody binding of immune cells and inducing B-cell tolerance in APLS, SLE and other diseases. However, no actual protection was demonstrated, and the disclosures emphasize the diagnostic use of the non immunogenic peptides. George J, et al (Atherosclerosis 1998;138:147-52) demonstrated the feasibility of immune suppression by hyperimmunization with MDA LDL and reduction of atherogenesis in mice. However, impractically large doses of antigen were required, and the paradoxical response to immunization with plaque antigens obviates the clinical efficacy of such therapy. Furthermore, none of the abovementioned studies disclose induction of mucosal tolerance for treatment of atherosclerosis.

Oral and mucosal tolerance for suppression and prevention of inflammatory conditions is well known in the art. Examples of candidate conditions, antigens and modes of therapy, can be found, for example in US Pat Nos. 5,935,577; 5,397,771; 4,690,683 to Weiner et al., and International Pat Nos. EP 0886471 A1 and WO 01821951 to Haas, et al. US Pat Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999, which is

incorporated herein in its entirety, teaches the oral administration of plaque associated antigens for the induction of tolerance in LDL receptor deficient mice. Measuring arterial fatty streak lesion density, the inventors demonstrated that oral administration of oxidized LDL, Beta2GPI and HSP 65 derived from animal sources were each able to produce approximately 30% reduction in atherogenesis. Typically, however, oral administration of antigens presents numerous obstacles to achieving tolerance and accurate dosing: the antigens are acted upon by digestion, altering both concentrations and molecular structure prior to their presentation to the lymphatic tissue of the Peyer's patches. Furthermore, the authors failed to investigate the efficacy of other routes of administration for induction of tolerance, such as mucosal and nasal tolerance. As the above mentioned disclosures clearly demonstrate, the parameters for induction of oral and mucosal tolerance cannot be deduced from antigenic activity in conventional immunization, or even in-vitro results, and must result from extensive empirical experimentation. Indeed, many studies have demonstrated the complexities inherent in manipulating the "balance between reacting and nonreacting" in the immune system. Zivny, et al (Clin Immunol 2001;101:150-68) clearly state that "In general, the response to one (tolerance inducing) antigen could not necessarily predict the response to another". Likewise, Hannihen et al (Diabetes 2001;50:771-75) observed that oral, nasal and respiratory administration of antigens caused appearance of disease symptoms (diabetes), rather than inducing tolerance. Similar inconsistencies in mucosal tolerance have been reported by Fujihashi et al (Acta Odontol Scand 2001;59:301-08), Jiang HR et al (Br J Ophthalmol 2001;85:739-44). Problems in mucosal vaccination strategies have been recently reviewed (Ogra PL, et al, Clin Microbiol Rev 2001;14:430-45; Chen H et al, J Control Release 2000;67:117-28; and Lehner T et al, J Infect Dis 1999;179 Suppl 3:S489-92).

While reducing the present invention to practice, the present inventors have uncovered that nasal administration of plaque associated molecules

results in the induction of mucosal tolerance, suppression of anti-plaque related antigen immune reactivity and protection from atherosclerosis. Mucosal tolerance is advantageous for its greater ease of application, accuracy of dosage and greatly reduced incidence of alteration of the tolerizing molecule by digestive and metabolic processes (especially in non-oral routes of administration). These advantages provide superior protection from atherogenic processes, improved patient compliance and reduced cost of therapy.

Thus, according to one aspect of the present invention there is provided a method of inducing immune tolerance to plaque associated molecules in a subject such as a human being.

The method, according to this aspect of the present invention is effected by administering to a subject (e.g., a human) a therapeutically effective amount of an antigenic portion of at least one plaque associated molecule.

As used herein, the phrase "mucosal administration" is defined as application of any and all compounds and/or compositions to mucosal membranes having component or components of the mucosal associated lymphatic tissue. Non-limiting examples of mucosal administration are buccal, intranasal, otic (middle ear), conjunctival, vaginal, rectal, etc. Mucosal administration excludes, for example, intravenous, subcutaneous and epidural administration.

As used herein, the phrase "plaque associated molecules" is defined as any and all protein, carbohydrate, lipid and nucleic acid molecules, portions thereof (antigenic portions), their derivatives, or combinations thereof physically or functionally related to the etiology, pathogenesis, symptomatology and/or treatment of a plaque related condition or disease. Such molecules may be, for example, plaque components such as oxidized LDL, foam cell components, etc, but may also include humoral and cellular entities, such as antibodies, cytokines, growth factors and T cell receptors.

As used herein, the phrase "antigenic portion" refers to a portion of a molecule capable of eliciting an immune response. For example, in cases where the molecule is a protein (e.g., HSP 65, Beta2GPI) such a portion can include a stretch of 6-8 amino acids that constitute an antigenic epitope. Methods for predicting antigenic portions are well known in the art, for example, DNASTAR'S PROTEAN sequence analysis and prediction module (DNAStar, Madison, WI). As such determining antigenic portions of plaque associated molecules suitable for use with the present invention is well within the capabilities of an ordinarily skilled artisan.

Plaque associated molecules (as well as fragments, analogs, portions and derivatives thereof) can be purified from natural sources (the tissue or organ where they normally occur) and can also be obtained using recombinant DNA technology, in bacterial, yeast, insect (e.g. baculovirus) and mammalian cells using techniques well-known to those of ordinary skill in the art. Amino acid sequences for many potential and actual plaque associated molecules are known, for example: human Beta2GPI (Accession No AAB21330 to Matsuura, et al), HSP65 (Accession No. AF65546 to Oliviera, et al) and human macrophage LDL scavenger receptor (Accession No. XP_008489 to NCBI Annotation Project).

Immune tolerance established using the present methodology can be used in the prevention and/or treatment of disorders associated with plaque formation, including but not limited to atherosclerosis, atherosclerotic cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and in-stent-stenosis. Some non-limiting examples of atherosclerotic cardiovascular disease are myocardial infarction, coronary arterial disease, acute coronary syndromes, congestive heart failure, angina pectoris and myocardial ischemia. Some non-limiting examples of peripheral vascular disease are gangrene, diabetic vasculopathy, ischemic bowel disease, thrombosis, diabetic retinopathy and diabetic nephropathy. Non-limiting examples of cerebrovascular disease are stroke, cerebrovascular inflammation,

cerebral hemorrhage and vertebral arterial insufficiency. Stenosis is occlusive disease of the vasculature, commonly caused by atheromatous plaque and enhanced platelet activity, most critically affecting the coronary vasculature. Restenosis is the progressive re-occlusion often following reduction of occlusions in stenotic vasculature. In cases where patency of the vasculature requires the mechanical support of a stent, in-stent-stenosis may occur, re-occluding the treated vessel.

Several plaque associated molecules are suitable for use with the present method. Examples include, but are not limited to, modified lipids, phospholipids and lipoproteins, apolipoprotein-lipid complexes such as LDL-cardiolipin, specific epitopes of proteinaceous molecules such as HSP and Beta2GPI, foam cell surface antigens such as LDL receptor and smooth muscle components such as troponin.

According to a preferred embodiment of the present invention the plaque associated molecule(s) utilized by the method of the present invention is oxidized LDL, Beta2GPI, HSP 65 and/or derivatives thereof.

According to another preferred embodiment of the present invention, a combination of at least two of the abovementioned molecules is administered to the subject.

The method of the invention may be used for prevention and/or treatment of non-atherosclerosis related diseases. For example, phospholipids, phospholipid metabolites and HSP 65 have been clearly implicated in the pathogenesis, and therefore potential treatment of additional, non-atherosclerosis-related diseases. Such diseases and syndromes include oxidative stress of aging (Onorato JM, et al, Annal N Y Acad Sci 1998 Nov 20;854:277-90), rheumatoid arthritis (RA)(Paimela L, et al. Ann Rheum Dis 1996 Aug;55(8):558-9), juvenile rheumatoid arthritis (Savolainen A, et al, 1995;24(4):209-11), inflammatory bowel disease (IBD)(Sawai T, et al, Pediatr Surg Int 2001 May;17(4):269-74), renal cancer (Noguchi S, et al, Biochem Biophys Res Commun 1992 Jan 31;182(2):544-50), venous and arterial

thromboses (Cabral AR, et al Am J Med 1996;101:472-81), Anti Phospholipid Syndrome (APLS or APS) (Koike T, et al Ann Med 2000;32 Suppl I:27-31), Systemic Lupus Erythematosus (US Pat Nos. 5,344,758 and 6,207,160, to Krilis, et al and Victoria, et al, respectively). Thus, the method of the invention may be used for prevention and/or treatment of non-atherosclerosis related diseases such as aging, RA, juvenile RA, IBD, SLE, APLS, thrombosis and cancer.

The immune tolerance inducing molecules or molecule combinations described hereinabove can be administered *per se*, or in a pharmaceutical composition where they are mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of the pharmaceutical composition of the present invention is to facilitate mucosal administration of the immune tolerance inducing molecules to an organism.

Herein the term "active ingredient" refers to the at least an antigenic portion of the plaque associated molecules (e.g. oxidized LDL, HSP 65, and beta2GP-I)or combinations thereof which are accountable for the biological effect (immune tolerance).

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of mucosal administration may, for example, include rectal, buccal, vaginal and especially transnasal, otic, conjunctival and respiratory (including intratracheal) application.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the mucosal route of administration chosen.

The active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. Penetrants appropriate to the barrier to be permeated may used in the formulation. Such penetrants are generally known in the art.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition of the present invention may be administered to the membranes of the eye, such as the conjuctiva. As such, the composition may be formulated in a liquid or semi-liquid composition, as described above, for application using for example, a drop applicator. Sterility may be ensured by sterilization methods known to one skilled in the art.

The pharmaceutical composition of the present invention may be formulated in rectal and vaginal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

The pharmaceutical composition of the present invention may be administered by inhalation. Examples of formulations for tolerizing agents that are administered by inhalation are provided in PCT/US90/07455, filed Dec. 17, 1990. The pharmaceutical formulations for administration by inhalation of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing and emulsifying agents, and salts of the type that are well-known in the art. Examples of such substances include normal saline solutions, such as physiologically buffered saline solutions, and water.

The route of administration of tolerizing antigens according to this alternate embodiment of the present invention is in an aerosol or inhaled form. The antigens can be administered as dry powder particles or as an atomized aqueous solution suspended in a carrier gas (e.g. air or N_2). Preferred aerosol pharmaceutical formulations may comprise for example, a physiologically-acceptable buffered saline solution containing between about 1 mg and about 300 mg of the antigens.

Dry aerosol in the form of finely divided solid particles of tolerizing antigens that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. The tolerizing antigens may be in the form of dusting powders and comprise finely divided particles having an average particle size of between about 1 and 5 microns, preferably between 2 and 3

microns. Finely divided particles may be prepared by pulverization and screen filtration using techniques well known in the art. The particles may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a powder.

Specific non-limiting examples of the carriers and/or diluents that are useful in the by-inhalation pharmaceutical formulations include water and physiologically-acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0. Additional non-limiting examples of suitable carriers or diluents for use in by-inhalation pharmaceutical formulations or dosage forms of the present invention are disclosed in U.S. Pat. No. 5,935,577 to Weiner, et al.

The pharmaceutical formulations of the present invention may be administered in the form of an aerosol spray using for example, a nebulizer such as those described by Weiner, et al (US Pat No. 5,935,577). The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S. P. in Aerosols and the Lung, Clarke, S. W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984, can be used when practicing the present invention. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, N.J.) and American Pharmoseal Co. (Valencia, Calif.).

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to induce an immune response thus preventing, alleviating or ameliorating symptoms of a disorder (e.g., atherosclerosis).

Ascertaining the optimum regimen for administering the active ingredient(s) is determined in light of the information disclosed herein and well known information concerning administration of mucosally active antigens, and autoantigens. Routine variation of dosages, combinations, and duration of treatment is performed under circumstances wherein the severity of atheromatous development can be measured. Useful dosage and administration parameters are those that result in reduction in inflammatory reaction, including a decrease in number of autoreactive T-cells, or in the occurrence or severity of at least one clinical or histological symptom of the disease.

The pharmaceutical composition of the present invention can be formulated comprising a therapeutically effective amount of additional compound or compounds useful in treating or preventing plaque related disease. In one preferred embodiment the additional compounds are HMGCoA reductase inhibitors (statins), mucosal adjuvants (see, for example, US Pat No. 6,270,758 to Staats, et al), corticosteroids, anti-inflammatory compounds (see, for example US Pat No. 6,297,260 to Bandarage, et al), analgesics, growth factors, toxins and additional tolerizing agents. In addition, it will be appreciated that use of the methods and compositions of the present invention does not preclude the initiation or continuation of other therapies for the abovementioned diseases or conditions, except where of specifically counterindicated.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

In further preferred embodiments of the present invention, cytokine and non-cytokine synergists can be conjoined in the treatment to enhance the effectiveness of mucosal tolerization with plaque associated molecules. Oral and parenteral use of other cytokine synergists (Type I interferons) has been

described in PCT/US95/04120, filed Apr. 07, 1995. Administration of Th2 enhancing cytokines is described in PCT application no. PCT/US95/04512, filed Apr. 07, 1995. For example, IL-4 and IL-10 can be administered in the manner described in PCT/US95/04512.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide mucosal levels of the active ingredient that are sufficient to induce tolerance. The "tolerizing dosage" will vary for each preparation, but can be estimated from *in vitro* data. Dosages necessary to achieve tolerizing dosage will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise an inhaler. The pack or inhaler may be

accompanied by instructions for administration. The pack or inhaler may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

It will be appreciated that administration of the tolerizing compositions and methods of the present invention may be effected by additional non mucosal methods such as intradermal, subcutaneous and intraperitoneal application.

According to another aspect of the present invention, there is provided an assay for determining the effects of mucosal administration of plaque associated molecules on artherosclerosis related disease or condition, the assay effected by mucosally administering the plaque associated molecule or composition thereof to a subject having such a disease or condition, and assessing at least one indicator of atherogenesis or inflammation. In a preferred embodiment, the plaque associated molecule is oxidized LDL, beta-2-GPI, HSP and/or derivates thereof. In another embodiment at least an antigenic portion of at least one plaque asspciated molecule is administered mucosally, the plaque associated molecule being a naturally occurring or synthetic molecule.

Indicators of atherogenesis or inflammation that can be assessed in the context of the assay of the present invention are known to the art. Some non-limiting examples are histological methods such as fatty streak lesion count, and immunological methods such as Stimulation Index, as described herein in

Examples section that follows. Progression of atherosclerosis can be assessed, for example, in atherosclerosis-prone mice maintained on an atherogenic diet (see, for example, George J, et al Circulation 1999;99:2227-30). Inflammation can be assessed by cytological, immunological, biochemical, molecular and genetic techniques known in the art.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include biochemical and immunological techniques. Such techniques are thoroughly explained in the literature. See, for example, "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; and "Methods in Enzymology" Vol. 1-317, Academic Press; Marshak et al., all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The

procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Materials and Experimental Methods

Animals: Apo-E deficient mice used in these experiments are from the atherosclerosis prone strain C57BL/6J-Apo-E^{tm1unc}. Mice homozygous for the Apo-E^{tm1unc} mutations show a marked increase in total plasma cholesterol levels which is unaffected by age or sex. Fatty streaks in the proximal aorta are found at 3 months of age. The lesions increase with age and progress to lesions with less lipid but more elongated cells, typical of a more advanced stage of pre-atherosclerotic lesion.

Strain Development: The Apo-E^{tm1unc} mutant strain was developed in the laboratory of Dr. Nobuyo Maeda at University of North Carolina at Chapel Hill. The 129-derived E14Tg2a ES cell line was used. The plasmid used is designated as pNMC109 and the founder line is T-89. The C57BL/6J strain was produced by backcrossing the Apo-E^{tm1unc} mutation 10 times to C57BL/6J mice (11,12).

The mice were maintained at the Sheba Hospital Animal Facility (Tel-Hashomer, Israel) on a 12-hour light/dark cycle, at 22-24°C and fed a normal fat diet of laboratory chow (Purina Rodent Laboratory Chow No. 5001) containing 0.027% cholesterol, approximately 4.5% total fat, and water, ad libitum. "Western diet" (TD 96125, Harlan Teklad, 42% calories from fat, 43% from carbohydrates and 15% from protein) describes a standardized, high fat atherogenic diet.

Nasal Tolerance: Nasal tolerance was induced by intranasal administration of oxidized LDL, Beta2GPI or HSP65, in a total volume of 10 µl PBS. Intranasal administration was performed on mildly sedated mice (12-16 weeks old), each mouse receiving 3 doses of antigen per dose, in the indicated concentrations, every other day. Atherogenesis was induced by 5

weeks of a Western diet, initiated on the day following the last intranasal administration. Controls received equal amounts of BSA and/or PBS, as indicated, in an identical regimen. Plasma samples were obtained for assessment of cholesterol and triglyceride levels from all mice, and the mice were sacrificed for evaluation of atherosclerosis, as described hereinbelow, after 5 weeks Western diet.

Oral Tolerance: For comparison, oral tolerance to plaque associated molecules was induced by feeding 3 doses of antigen every other day (for a detailed account of induction of oral tolerance, see US Pat Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999), in a similar regimen to the nasal tolerance.

Antigen Preparation

Beta2GPI: Human Beta2GPI was purified from the serum of a healthy adult as described by Gharavi, et al (J Clin Invest 1992;92:1105-09).

Oxidized LDL: Human LDL (density=1.019- 1.063g/l) was prepared from Plasma of fasting individuals by preparative ultracentrifugation (50,000 rpm/min, 22 min), washing, dialysis against 150mM EDTA, pH 7.4, filtration (0.22 μm pore size) to remove aggregation, and storage under nitrogen. LDL oxidation was performed by incubation of dialyzed, EDTA-free LDL with copper sulfate (10 μM) for 24 hours at 37° C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (TBARS), which measures malondialdehyde (MDA) equivalents.

HSP65: Recombinant mycobacterial HSP-65, prepared as described (Prohaszka Z et al, Int Immunol 1999;11:1363-70) was kindly provided by Dr. M. Singh, Braunschweig, Germany.

Immunization: Subcutaneous immunization with human Beta2GPI: Human Beta2GPI was prepared from human plasma pool as described above. For immunization, human Beta2GPI was dissolved in PBS and mixed with equal volumes of Freund's incomplete adjuvant. Immunizations were performed by single subcutaneous injection of 10μg

antigen/mouse in 0.1ml volume. Three days following the last mucosal administration of plaque associated molecules the mice received one immunization, and were sacrificed 10 days post immunization.

Cholesterol Level Determination: At the completion of the experiment, 1-1.5 ml of blood was obtained by cardiac puncture into vials containing EDTA, centrifuged to separate plasma. Total plasma cholesterol levels were determined using an automated enzymatic technique (Boehringer Mannheim, Germany).

FPLC Analysis: Fast Protein Liquid Chromatography analysis of cholesterol and lipid content of lipoproteins was performed using Superose 6 HR 10/30 column (Amersham Pharmacia Biotech, Inc, Peapack, NJ) on a FPLC system (Pharmacia LKB. FRAC-200, Pharmacia, Peapack, NJ). A minimum sample volume of 300 µl (blood pooled from 3 mice was diluted 1:2 and filtered before loading) was required in the sampling vial for the automatic sampler to completely fill the 200 µl sample loop. Fractions 10-40 were collected, each fraction contained 0.5 ml. A 250 µl sample from each fraction was mixed with freshly prepared cholesterol reagent or triglyceride reagent respectively, incubated for 5 minutes at 37°C and assayed spectrophotometrically at 500nm.

Assessment of Atherosclerosis: Quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described (George J et al Circulation 1999;99:2227-30)and by calculating the lesion size in the aorta. Briefly, after perfusion with saline Tris EDTA, the heart and the aorta were removed from the animals and the peripheral fat cleaned carefully. The upper section of the heart was embedded in OCT medium (10.24% w/w polyvinyl alcohol; 4.26% w/w polyethylene glycol; 85.50% w/w nonreactive ingredients) and frozen. Every other section (10 µm thick) throughout the aortic sinus (400 µm) was taken for analysis. The distal portion of the aortic sinus was recognized by the three valve cusps that

are the junctions of the aorta to the heart. Sections were evaluated for fatty streak lesions after staining with oil-red O. Lesion areas per section were scored on a grid by an observer counting unidentified, numbered specimens. The aorta was dissected from the heart and surrounding adventitious tissue was removed. Fixation of the aorta and Sudan staining of the vessels were performed as previously described (George J et al Circulation 1999;99:2227-30).

Proliferation assays: Mice were exposed to the tested antigen as described for assessment of atherosclerosis, and then immunized (one to three days following the last exposure) subcutaneously with 10 μg Beta2GPI in 0.1 ml PBS, prepared from purified human Beta2GPI as described above.

Proliferation was assayed ten days after immunization with the Beta2GPI as follows: Draining inguinal lymph nodes were prepared by meshing the tissues on 100 mesh screens. Red blood cells were lysed with cold sterile double distilled water (6ml) for 30 seconds and 2ml of NaCl 3.5% was added. Incomplete medium was added (10ml), cells were centrifuged for 7 min at 1,700 rpm, resuspended in RPMI medium and counted in a haemocytometer at 1:20 dilution (10μl cells + 190μl Trypan Blue). Proliferation was measured by the incorporation of [^{3}H] Thymidine into DNA in triplicate samples of 100μl of the packed cells ($1x10^6$ cells/ml) in a 96 well microtiter plate. Triplicate samples of Beta2GPI (10μg/ml, 100μl/well) were added, cells incubated for 72 hours (37°C, 5% CO_2 and ~98% humidity) and 10μl ^{3}[H] Thymidine (0.5μCi/well) was added. After an additional day of incubation the cells were harvested and transferred to glass fiber filters using a cell harvester (Brandel) and counted using β-counter (Lumitron). Proliferation was measured by the incorporation of [^{3}H] thymidine into DNA during the final 12 h of incubation. The results are expressed as the stimulation index (S.I.): the ratio of the mean radioactivity (cpm) of the antigen to the mean

background (cpm) obtained in the absence of the antigen. Standard deviation was always <10% of the mean cpm.

Statistical Analysis: A one-way ANOVA test was used to compare independent values. $p<0.05$ was accepted as statistically significant.

EXAMPLE 1

Inhibition of atherogenesis in genetically predisposed (Apo-E-deficient) mice by induction of nasal tolerance with low doses of the plaque associated molecules oxidized LDL, human Beta2GPI and HSP 65

The present inventors here demonstrate, for the first time, that nasal exposure to low doses of the plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 provides induction of tolerance to the antigens, and significant inhibition of atherogenesis. Thus, nasal exposure to purified, oxidized human LDL, human Beta2GPI and recombinant mycobacterial HSP 65 were compared for their effectiveness in suppressing atherogenesis in Apo-E-deficient mice. 63 male 9-13 week old Apo E/C 57 mice were divided into 5 groups. In group A (HSP-65)(n=12) nasal tolerance was induced as described in Materials and Methods by administration of recombinant mycobacterial HSP 65 suspended in PBS (10 µg/mouse/10µl) for 5 days every other day. In group B (H-oxLDL)(n=14) nasal tolerance was induced as described in Materials and Methods by administration of 10 µg/mouse/10µl oxidized purified human LDL, suspended in PBS, every other day for 5 days. Mice in group C (B2GPI)(n=13) received 10 µg/mouse/10µl human Beta2GPI per mouse, administered intranasally as described in Materials and Methods, every other day for 5 days. Mice in group D (BSA)(n=12) received 10 µg/mouse/10µl bovine serum albumin (BSA) per mouse, administered intranasally as described in Materials and Methods, every other day for 5 days. Mice in group E (PBS)(n=12) received 10µl PBS per mouse, administered intranasally. Mice were bled prior to feeding (Time 0) and at the conclusion of

the experiment (End) for determination of lipid profile. Atherosclerosis was assessed in heart and aorta as described above, 8 weeks after the last feeding. Mice were weighed every 2 weeks during the experiment. All mice were fed water ad libitum and a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol), up to the final antigen exposure, and then a "Western" diet until sacrifice.

Table 1: Inhibition of atherogenesis in Apo-E-deficient mice by intranasal administration of exceedingly low doses of plaque associated molecules

		HSP-65	H-oxLDL	H-B2-GPI	BSA	PBS	
Time 0	Weight (gr) (Mean±S.E)	22.6 ±0.8	22.3 ±0.5	22.3 ±0.7	21.8 ±0.7	21.7 ±0.5	P=0.833
	Chol (mg/dL) (Mean±S.E)	237 ±13	230 ±10	230 ±14	236±19	227±14	P=0.986
	TG (mg/dL) (Mean±S.E)	150 ±19	178 ±17	162 ±18	185±22	160±15	P=0.664
END	Weight (gr) (Mean±S.E)	26.8 ±0.9	28.2 ±1.0	29.2 ±1.5	25.5±1.0	26.3±1.3	P=0.157
	Chol (mg/dL) (Mean±S.E)	1181 ±114	1611 ±119	1601 ±125	1470±183	1606 ±181	P=0.197
	TG (mg/dL) (Median)	288	275	380	315	403	P=0.416
	Sinus Lesion (μm^2) (Mean±S.E)	**44375 ±5437**	**43393 ±4107**	**46250 ±4486**	**120500 ±8746**	**128182 ±9102**	P<0.001

Note: "Weight" is weight in grams; "Chol" is serum cholesterol and "TG" is serum triglycerides, expressed in mg/dL.

As can be seen from Figure 1, the results depicted in Table 1 demonstrate the strikingly effective inhibition of atherogenesis measured in the tissues of mice receiving nasal exposure to low doses (10 μg/ mouse) of the plaque associated molecules, compared to control mice exposed to sham antigen (BSA) or PBS. Furthermore, nasal tolerance is specific in its mode of protection: clearly, induction of nasal tolerance has no significant effect on other general parameters measured, such as weight gain, triglyceride or cholesterol blood levels. Thus, the antigenic plaque associated molecules oxidized LDL, Beta2GPI and HSP 65 are highly potent inducers of nasal

tolerance, with surprisingly low doses (10 μg/ mouse) and brief exposure (3 days) of significant (greater than 65%) and consistent protection from atherogenesis in these genetically susceptible Apo-E-deficient mice.

EXAMPLE 2

Superior inhibition of atherogenesis in genetically predisposed (Apo-E-deficient) mice by induction of nasal tolerance with HSP 65

The present inventors here demonstrate, for the first time, that nasal exposure to exceedingly low doses of the plaque associated molecule HSP 65 provides superior induction of tolerance to the antigen, and inhibition of atherogenesis. Thus, nasal exposure to a low dose and an exceedingly low dose of recombinant human HSP 65 were compared for their effectiveness in suppressing atherogenesis in Apo-E-deficient mice. 58 male 12-16 week old Apo E/C 57 mice were divided into 4 groups. In group A (HSP-65 high)(n=14) nasal tolerance was induced as described in Materials and Methods by intranasal administration of 10μg/mouse/10μl recombinant human HSP 65 suspended in PBS for 5 days every other day. In group B (HSP-65 low)(n=16) nasal tolerance was induced as described in Materials and Methods by administration of 1 μg/mouse/10μl recombinant human HSP 65 suspended in PBS every other day for 5 days. Mice in group C (BSA)(n=14) received 1 μg/mouse/10μl BSA per mouse, administered intranasally, every other day for 5 days. Mice in group D (PBS)(n=14) received 10μl PBS per mouse, administered intranasally. Mice were bled prior to feeding (Time 0) and at the conclusion of the experiment (End) for determination of lipid profile. Atherosclerosis was assessed in heart and aorta as described above, 8 weeks after the last feeding. Mice were weighed every 2 weeks during the experiment. All mice were fed water ad libitum and a normal chow-diet containing 4.5% fat by weight (0.02% cholesterol), up to the final antigen exposure, and then a "Western" diet until sacrifice.

Table 2: Superior inhibition of atherogenesis in Apo-E-deficient mice by intranasal administration of human HSP 65

		HSP65 *10* *μg/Mouse* *N=12*	*HSP65* *1* *μg/Mouse* *N=16*	*BSA* *100* *μg/Mouse* *N=11*	PBS *N=10*	Statistics
End	Wt	28.4 ±1.0	26.9 ±0.9	27.7 ±0.5	28.7 ±0.7	P=0.363
	Chol	1073 ±65	1010 ±64	1009 ±74	1015 ±85	P=0.897
	TG	348 ±32	315 ±46	316 ±32	390 ±44	P=0.564
	Sinus Les. μm^2	**22292 ±2691**	**17109 ±2053**	**54432 ±8201**	**47750 ±5779**	**P<0.05 Between HSP-65 and PBS or BSA**

Note: "Weight" is weight in grams; "Chol" is serum cholesterol and "TG" is serum triglycerides, expressed in mg/dL.

As can be seen from Figure 2, the results depicted in Table 2 demonstrate the superior effectiveness of inhibition of atherogenesis measured in the tissues of mice receiving nasal exposure to exceedingly low doses (1 μg/ mouse) of HSP 65, compared to control mice exposed to sham antigen (BSA) or PBS. Furthermore, nasal tolerance is specific in its mode of protection: clearly, induction of nasal tolerance has no significant effect on other general parameters measured, such as weight gain, triglyceride or cholesterol blood levels. Thus, the antigenic plaque associated molecule HSP 65 is an extremely potent inducer of nasal tolerance, with even exceedingly low doses conferring significant (approximately 70%) protection from atherogenesis in genetically susceptible Apo-E-deficient mice, greatly superior to the protection achieved by induction of oral tolerance (30%; see US Patent Application No 09/806,400 to Shoenfeld et al filed Sept 30, 1999).

EXAMPLE 3

Superior suppression of specific anti-Beta2GPI immune reactivity in genetically predisposed (apo-E deficient) mice by intranasal administration of human Beta2GPI

Tolerance induced by mucosal exposure to plaque associated molecules may be mediated by suppression of specific immune responses to antigenic

portions of these plaque associated molecules. Lymphocyte proliferation in response to mucosal (nasal and oral) exposure to human Beta2GPI was measured in Apo-E-deficient mice. 9 male, 5 week old Apo E/C 57 deficient mice were divided into 3 groups. In group A (n=3) oral tolerance was induced with 100 μg/mouse Beta2GPI suspended in 0.2 ml PBS, administered by gavage, as described above, every other day for 5 days. In group B (n=3) nasal tolerance was induced with 10 μg/mouse Beta2GPI suspended in 10 μl PBS, administered intranasally as described above, every other day for 5 days. The mice in group C (n=3) received oral administration of 200 μl PBS every other day for 5 days. Immune reactivity was stimulated in all mice by immunization with human Beta2GPI as described above in the Materials and Methods section, one day after the last feeding. Ten days after the immunization lymph nodes were collected for assay of proliferation. All mice were fed normal chow-diet containing 4.5% fat by weight (0.02% cholesterol) and water ad libitum.

Table 3: Intranasal pretreatment with purified human beta2 GPI suppresses immune response to Human Beta2GPI in Apo-E-deficient mice

	PBS	H-β_2-GPI OT	H-β_2-GPI NT
S.I (Stimulation Index)	7.0±0.2	4.4±0.5	2.1±0.5

As can be seen from Figure 3, the results depicted in Table 3 demonstrate significant suppression of immune reactivity to human Beta2GPI antigen, measured by inhibition of proliferation in the lymph nodes of Apo-E-deficient mice. Lymphocytes from mice receiving intranasal exposure to low atherogenesis-inhibiting doses (10 μg/ mouse) of human Beta2GPI showed an exceedingly reduced stimulation index following immunization with Beta2GPI, as compared to orally exposed and control (PBS) mice. Since previous studies with induction of nasal tolerance have shown no significant

effect on other parameters measured, such as weight gain, triglyceride or cholesterol blood levels, or immune competence (see abovementioned Examples), these results indicate a specific suppression of anti-Beta2GPI immune reactivity. Thus, intranasal administration of the purified plaque associated molecule Beta2GPI is a superior method of attenuating the cellular immune response to immunogenic and atherogenic plaque associated molecules in these genetically susceptible Apo-E-deficient mice.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents, patent applications and sequences identified by their accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent, patent application or sequence identified by their accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition for prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, comprising, as an active ingredient, a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule, or pharmaceutical salts thereof, the composition further comprising a pharmaceutically acceptable carrier, the pharmaceutical composition being designed for mucosal administration.

2. The composition of claim 1, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.

3. The composition of claim 1, wherein said antigenic portion of at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.

4. The composition of claim 1, wherein the pharmaceutical composition is formulated for nasal, respiratory, otic and/or conjuctival administration.

5. The composition of claim 1, wherein said at least said antigenic portion of said at least one plaque associated molecule is selected so as to reduce immune reactivity to plaque components in the subject.

6. The composition of claim 1, packaged and identified for use in the prevention and/or treatment of at least one disorder selected from the group

consisting of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, stenosis, restenosis and/or in-stent-stenosis.

7. The composition of claim 1, further comprising a therapeutically effective amount of at least one additional compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

8. An assay for determining the effect of mucosal administration of plaque components on atherosclerosis-related disease or condition, the assay comprising:

(a) mucosally administering to a subject having an atherosclerosis-related disease or condition at least an antigenic portion of at least one plaque associated molecule and,

(b) assessing at least one indicator of atherogenesis in said subject to thereby determine the effect of mucosal administration of said at least said antigenic portion of said at least one plaque associated molecule on the atherosclerosis-related disease or condition .

9. The assay of claim 8, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.

10. The assay of claim 8, wherein said antigenic portion of said at least one plaque associated molecule is a naturally occurring molecule or a synthetic molecule.

11. A method of prevention and/or treatment of atherosclerosis, cardiovascular disease, cerebrovascular disease, peripheral vascular disease,

stenosis, restenosis and/or in-stent-stenosis in a subject in need thereof, the method comprising mucosally administering a therapeutically effective amount of at least an antigenic portion of at least one plaque associated molecule to the subject.

12. The method of claim 11, wherein said plaque associated molecule is selected from the group consisting of oxidized LDL, beta-2-GPI, HSP and derivatives thereof.

13. The method of claim 11, wherein said at least said antigenic portion of at least one plaque associated molecule is a naturally occurring or synthetic molecule.

14. The method of claim 11, wherein administration of said antigenic portion of said at least one plaque associated molecule is effected via nasal, respiratory, otic and/or conjunctival route.

15. The method of claim 11, wherein administration of said at least said antigenic portion of at least one plaque associated molecule reduces immune reactivity to said at least one plaque associated molecule in said subject.

16. The method of claim 11, wherein said at least said antigenic portion of said at least one plaque associated molecule is administered along with a therapeutically effective amount of a compound selected from the group consisting of HMGCoA reductase inhibitors (statins), mucosal adjuvants, corticosteroids, anti-inflammatory compounds, analgesics, growth factors, toxins, and additional tolerizing antigens.

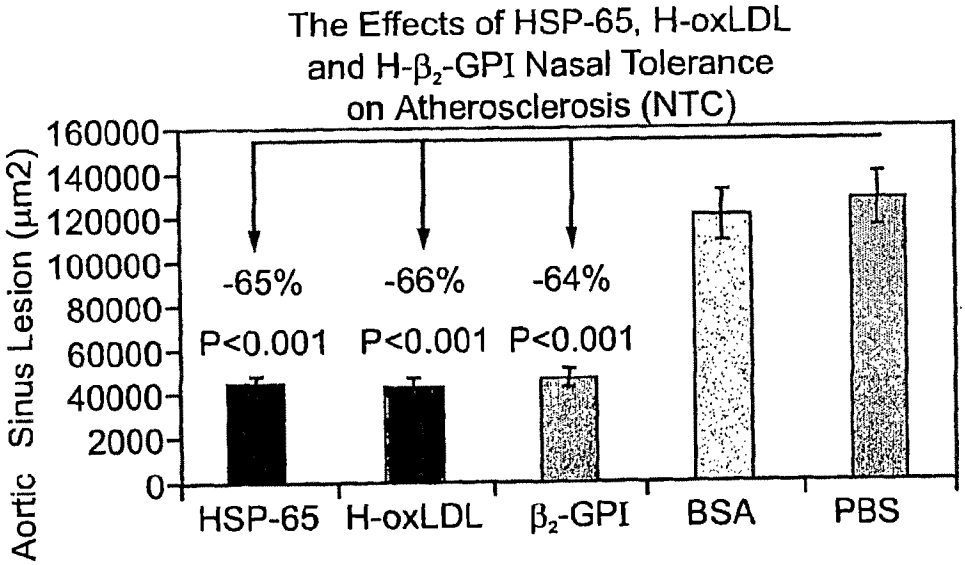
The Effects of HSP-65, H-oxLDL
and H-β2-GPI Nasal Tolerance
on Atherosclerosis (NTC)
Aortic Sinus Lesion (µm2)
160000
140000
120000
100000
80000
60000
40000
2000
0
-65%
-66%
-64%
P<0.001
P<0.001
P<0.001
HSP-65
H-oxLDL
β2-GPI
BSA
PBS

Fig. 1

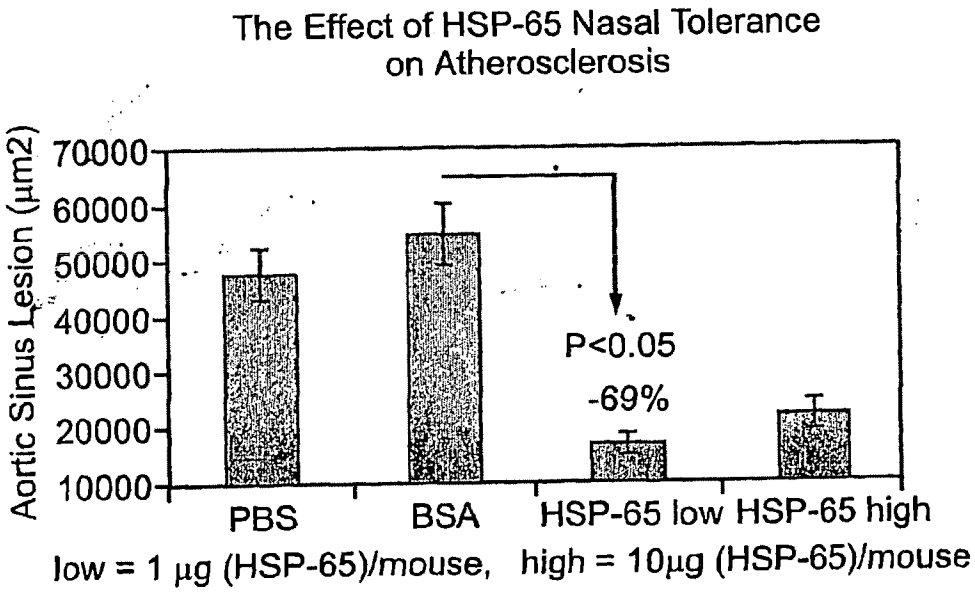
The Effect of HSP-65 Nasal Tolerance
on Atherosclerosis
Aortic Sinus Lesion (µm2)
70000
60000
50000
40000
30000
20000
10000
P<0.05
-69%
PBS
BSA
HSP-65 low
HSP-65 high
low = 1 µg (HSP-65)/mouse, high = 10µg (HSP-65)/mouse

Fig. 2

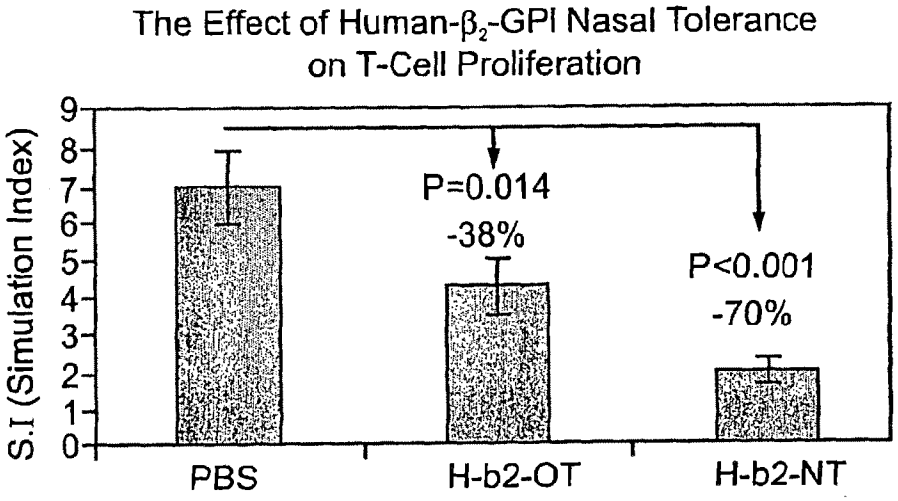
The Effect of Human-β2-GPI Nasal Tolerance
on T-Cell Proliferation
S.I (Simulation Index)
9
8
7
6
5
4
3
2
1
0
P=0.014
-38%
P<0.001
-70%
PBS
H-b2-OT
H-b2-NT

Fig. 3

EXHIBIT J

Effects of Oral Insulin in Relatives of Patients With Type 1 Diabetes

The Diabetes Prevention Trial–Type 1

THE DIABETES PREVENTION TRIAL–TYPE 1 STUDY GROUP

OBJECTIVE — This randomized, double-masked, placebo-controlled clinical trial tested whether oral insulin administration could delay or prevent type 1 diabetes in nondiabetic relatives at risk for diabetes.

RESEARCH DESIGN AND METHODS — We screened 103,391 first- and second-degree relatives of patients with type 1 diabetes and analyzed 97,273 samples for islet cell antibodies. A total of 3,483 were antibody positive: 2,523 underwent genetic, immunological, and metabolic staging to quantify risk of developing diabetes; 388 had a 5-year risk projection of 26–50%; and 372 (median age 10.25 years) were randomly assigned to oral insulin (7.5 mg/day) or placebo. Oral glucose tolerance tests were performed every 6 months. The median follow-up was 4.3 years, and the primary end point was diagnosis of diabetes.

RESULTS — Diabetes was diagnosed in 44 oral insulin and 53 placebo subjects. Annualized rate of diabetes was similar in both groups: 6.4% with oral insulin and 8.2% with placebo (hazard ratio 0.764, $P = 0.189$). In a hypothesis-generating analysis of a subgroup with insulin autoantibody (IAA) levels confirmed (on two occasions) ≥80 nU/ml ($n = 263$), there was the suggestion of benefit: annualized diabetes rate 6.2% with oral insulin and 10.4% with placebo (0.566, $P = 0.015$).

CONCLUSIONS — It is possible to identify individuals at high risk for type 1 diabetes and to enroll them in a large, multisite, randomized, controlled clinical trial. However, oral insulin did not delay or prevent type 1 diabetes. Further studies are needed to explore the potential role of oral insulin in delaying diabetes in relatives similar to those in the subgroup with higher IAA levels.

Diabetes Care 28:1068–1076, 2005

The Diabetes Prevention Trial–Type 1 (DPT-1) was a randomized controlled clinical initiative designed to determine whether it is possible to prevent or delay the onset of overt diabetes in relatives of patients with type 1 diabetes. DPT-1 included two separate trials. Relatives were screened for islet cell antibodies (ICAs), and those who were positive underwent further testing to assess projected 5-year risk of diabetes. Earlier we reported the results of the DPT-1 parenteral insulin trial, conducted in relatives with >50% projected 5-year risk of diabetes (1). This article reports the results of the DPT-1 oral insulin trial in relatives with a projected 5-year risk of diabetes of 26–50%. In both trials, relatives were studied because of their 10- to 20-fold increased risk compared with the general population (2,3).

Type 1 diabetes is a consequence of immune-mediated destruction of insulin-secreting pancreatic islet β-cells (4). A number of studies have suggested that oral administration of autoantigens induces protective immunity that has the potential to downregulate ongoing destructive immune reactions (5–7). Peptides derived from an orally administered antigen encounter the mucosal gut–associated lymphoid tissue, which serves both to protect the host from ingested pathogens and to prevent the host from reacting to ingested proteins. The concept is that low doses of orally administered autoantigens suppress autoimmunity by inducing antigen-specific regulatory T-cells in the gut, which act by releasing inhibitory cytokines at the target organ (5–7). In the mid-1990s, the concept of oral antigen administration was quite popular, and studies were initiated in a number of human autoimmune diseases. In the nonobese diabetic mouse model of type 1 diabetes, oral administration of insulin to young, pre-diabetic mice inhibits their development of type 1 diabetes (8–13). Oral insulin also prevented diabetes and even reversed hyperglycemia in a transgenic mouse model of virus-induced diabetes (14). The results in these animal models suggested that oral insulin could attenuate pancreatic islet autoimmunity, leading to a delay in the onset of the disease, and was the impetus to conduct the DPT-1 oral insulin trial. Moreover, the breakdown of insulin into smaller peptides in the gastrointestinal tract would avoid any hypoglycemic effects of insulin,

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Jay S. Skyler, MD (DPT-1 Study Chairman), takes full responsibility for the analysis and integrity of the data and the writing of the manuscript.

Additional information for this article can be found in an online appendix at http://care.diabetesjournals.org.

Abbreviations: DPT-1, Diabetes Prevention Trial–Type 1; FPG, fasting plasma glucose; FPIR, first-phase insulin response; IAA, insulin autoantibody; ICA, islet cell antibody; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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an additional potential benefit for testing oral insulin.

RESEARCH DESIGN AND METHODS

The study was divided into three parts: screening, staging, and intervention (1). Participants were recruited from study clinics and through media campaigns.

Screening

First-degree (ages 3–45 years) and second-degree (ages 3–20 years) relatives of patients with type 1 diabetes were screened for ICAs. Those with ICA titer ≥10 Juvenile Diabetes Foundation units were invited to have staging evaluations.

Staging

Staging confirmed ICA positivity, measured insulin autoantibody (IAA) status, assessed first-phase insulin response (FPIR) to intravenous glucose, assessed oral glucose tolerance (OGT), and determined presence or absence of HLA-DQA1*0102/DQB1*0602 (a protective haplotype that excluded subjects from participation) (15,16). Relatives who were ICA$^+$ and IAA$^+$ and with FPIR above threshold (defined below) and normal glucose tolerance were projected to have a 5-year risk of 26–50% ("intermediate risk") and were eligible for the oral insulin trial. Those identified as having a 5-year risk of ≥50% ("high risk") were eligible for the parenteral insulin trial previously reported (1). The original protocol had an entry criterion of confirmed (on two occasions) IAA level >5 SD above the mean of the normal reference range (i.e., ≥80 nU/ml). In October 1997, after review of data from natural history studies suggesting that a sufficient cutoff was >3 SD above the mean of the reference range, to enhance enrollment the entry criterion was changed to that level (i.e., IAA ≥39 nU/ml).

Intervention

The study was a double-masked, placebo-controlled, randomized clinical trial, in which participants were assigned to receive capsules of either oral insulin, 7.5 mg of recombinant human insulin crystals (Eli Lilly, Indianapolis, IN), or matched placebo. Capsules were prepared with methylcellulose filler at a compounding pharmacy (Belmar Pharmacy, Lakewood, CO). Masked bottles of oral insulin or placebo were shipped to clinical sites from a research pharmacy (Moffitt Cancer Center, Tampa, FL). Subjects consumed the capsule as a single daily dose before breakfast each day, either by taking the capsule or, if the subject could not swallow capsules, sprinkling its contents in juice or on food. Randomization used a central automated system, stratified by clinical center, using random variable block sizes.

Study sites

Study coordination, laboratory tests, and data management were done centrally. Protocols were approved by institutional review boards at all participating locations across the U.S. and Canada, including 91 sites conducting the intervention. Participants (and/or their parents) provided separate written consent for each part, screening, staging, and intervention, and yearly thereafter for continuation in the study.

Role of the funding source

Representatives from the sponsoring institutes of the National Institutes of Health (National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Allergy and Infectious Diseases, and National Institute of Child Health and Human Development) served on the Steering Committee and virtually all of the study group committees and were full participants on their committees, which were involved in all aspects of protocol design, data analysis, and preparation of the manuscript. The other funding sources provided only resources and were not involved in the study per se.

Follow-up assessments

Participants were seen every 6 months, and at those visits an OGT test was performed to assess glycemic status, the primary study end point. An intravenous glucose tolerance test was performed at baseline, annually thereafter, and at study end. Mixed-meal tolerance tests were performed at baseline, after 3 years, and at study end.

Participants checked blood glucose if they experienced symptoms of hypoglycemia. Presumed hypoglycemia (without measurement of glucose) was defined as typical symptoms that promptly resolved with food intake. Definite hypoglycemia was defined as blood glucose <2.8 mmol/l (50 mg/dl) measured at the time of symptoms. Severe hypoglycemia was defined as loss of consciousness, convulsion, stupor, or hypoglycemia requiring assistance of another person or treatment with intravenous glucose or subcutaneous glucagon. Chemical hypoglycemia was defined by five-point (before breakfast, before lunch, before supper, 2 h after supper, 3:00 A.M.) home capillary blood glucose profiles obtained quarterly, if two of these glucose values were <2.8 mmol/l (<50 mg/dl).

Tolerance test procedures

Tolerance tests were performed after an overnight fast. Samples were drawn through a temporary indwelling intravenous catheter. Intravenous glucose tolerance tests were performed as described (17,18). Insulin values at 1 and 3 min were added to determine FPIR. FPIR was above threshold if ≥10th percentile for siblings, offspring, and second-degree relatives (≥100 μU/ml if age ≥8 years; ≥60 μU/ml if age <8 years) and ≥1st percentile for parents (≥60 μU/ml). FPIR above threshold was required for eligibility.

For the oral glucose tolerance test, the oral glucose (Sundex, Fisher) dose was 1.75 g/kg (maximum 75 g). Plasma glucose values were interpreted according to American Diabetes Association guidelines (19): fasting plasma glucose (FPG) ≥7.0 mmol/l (≥126 mg/dl) or 120-min glucose ≥11.1 mmol/l (≥200 mg/dl) was considered diagnostic of diabetes; FPG 6.1–6.9 mmol/l (110–125 mg/dl) signified impaired fasting glucose (IFG); 120-min glucose 7.8–11.1 mmol/l (140–199 mg/dl) signified impaired glucose tolerance (IGT). If a 30-, 60-, or 90-min level was ≥11.1 mmol/l (≥200 mg/dl) but FPG and 120-min levels were below threshold for IFG and IGT, this was noted as indeterminate glucose tolerance. A normal OGT during staging was required for eligibility. Diagnosis of diabetes required confirmation on a subsequent day by OGT, elevated fasting plasma glucose, or random plasma glucose ≥11.1 mmol/l (≥200 mg/dl) accompanied by symptoms of polyuria, polydipsia, and/or weight loss (19).

For the mixed-meal tolerance test, a liquid formula meal was consumed (Sustacal/Boost, Mead Johnson Nutritionals; 6 kcal/kg body weight, maximum 360 kcal).

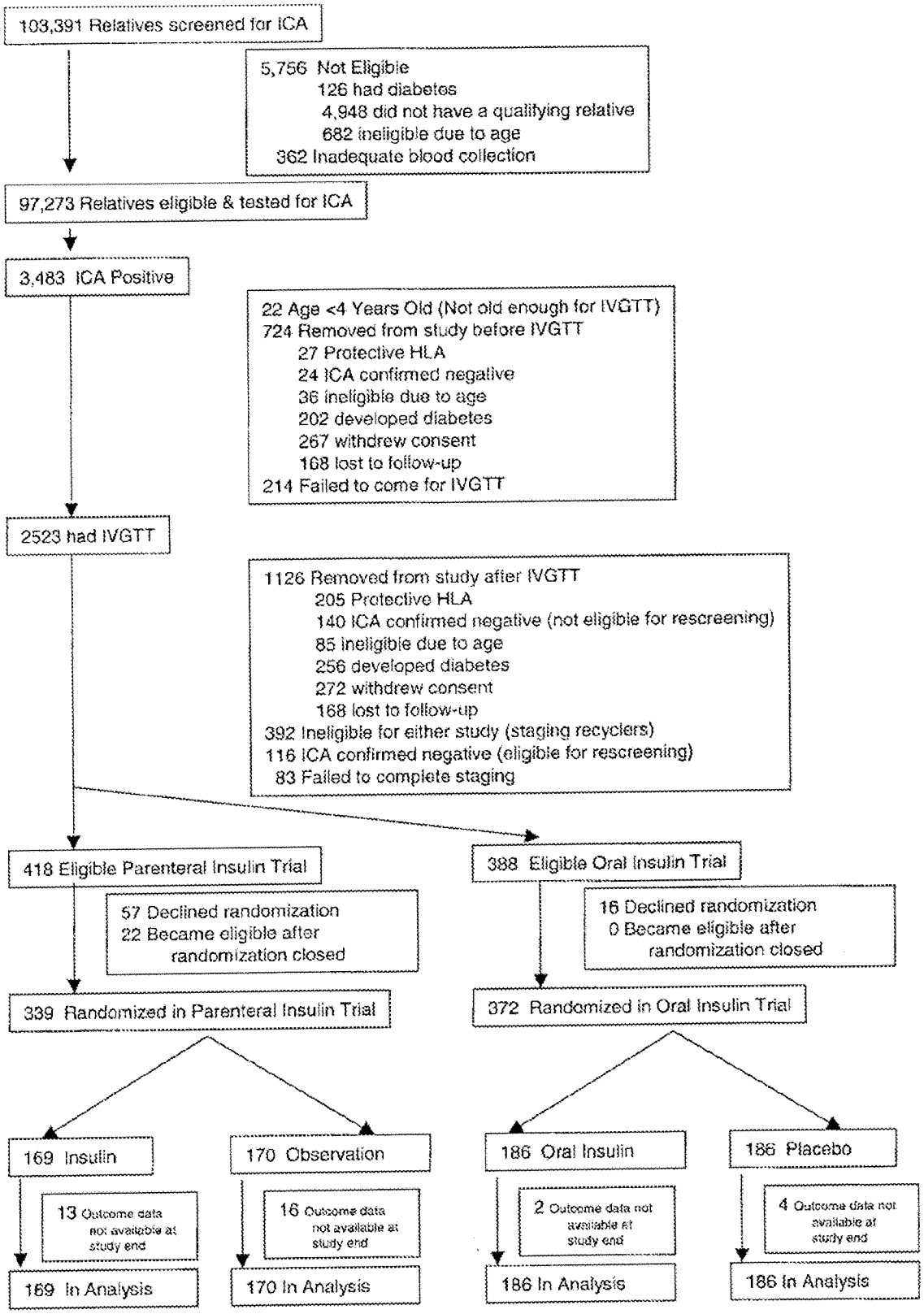

Figure 1—*Flow diagram of all subjects recruited to trial. IVGTT, intravenous glucose tolerance test.*

Table 1—*Baseline characteristics of randomly assigned subjects*

	Oral insulin group	Placebo group	*P* value
n	186	186	
Median age	11.0 (7–14)	9.8 (7–14)	0.3569
Average FPIR (μU/ml)	161.6 ± 72.4	158.9 ± 99.2	0.7672
Race			0.2897
White	164 (86.1)	163 (87.6)	
African American	5 (2.6)	2 (1.0)	
Hispanic	8 (4.3)	14 (7.5)	
Other	9 (4.7)	7 (3.7)	
Sex			0.1381
Male	119 (63.9)	105 (56.4)	
Female	67 (36.0)	81 (43.5)	
Relationship to index patient with diabetes			0.6552
Sibling	112 (60.2)	108 (58.0)	
Offspring	49 (26.3)	53 (28.4)	
Parent	11 (5.9)	7 (3.7)	
Second degree	14 (7.5)	18 (9.6)	
Antibody levels			
Median ICAs (JDF units)	80 (40–200)	80 (40–160)	0.9253
Mean IAAs (nU/ml)	382 ± 555	346 ± 436	0.4910
GAD antibodies			0.2908
Positive	144 (77.8)	136 (66.4)	
Negative	41 (22.1)	50 (43.5)	
ICA-512 antibodies			0.9567
Positive	97 (52.4)	97 (52.1)	
Negative	88 (47.5)	89 (47.8)	
Micro IAA			0.0551
Positive	39 (29.3)	28 (19.4)	
Negative	94 (70.6)	116 (80.5)	
HbA_{1c} (%)	5.35 ± 0.39	5.33 ± 0.34	0.5949
C-peptide area under curve			
During intravenous glucose tolerance test	34.8 (15.6)	35.1 (16.7)	0.8800
During oral glucose tolerance test	502.5 (201.3)	502.1 (207.2)	0.9858
During mixed meal tolerance test	383.1 (172.4)	381.0 (183.9)	0.9102

Data are means ±SD, *n* (%), or mean (interquartile range).

Laboratory measures

All assays were performed as previously described (1), including ICA (indirect immunofluorescence), IAA (competitive fluid-phase radioassay), plasma glucose (glucose oxidase method), insulin (radioimmunoassay), C-peptide (radioimmunoassay), and HLA-DQ typing (PCR using sequence-specific probes).

Statistical methods

The trial was designed assuming a 5-year cumulative diabetes incidence of 26–50% (annual hazard rate 6%), 80% power to detect a 50% reduction in incidence in the oral insulin group, and $\alpha = 0.05$ (two-tailed). The oral insulin trial was designed to accrue subjects for 4 years with 2 years of follow-up and an annual rate of loss to follow-up of 10%, yielding an estimated average planned duration of treatment of 2.8 years with a projected 70 events occurring. The original projection was for a 4-year accrual period and sample size of 490 subjects in the oral insulin trial.

Variables not normally distributed were log-transformed for analysis and back-transformed for presentation. Data were analyzed according to the intention-to-treat principle. Kaplan-Meier life tables were constructed and compared by the log-rank χ^2 statistic. Categorical variables were compared by Pearson's χ^2 test or Fisher's exact test. Differences in means were tested using ANOVA. Tests of significance were two-tailed. Statistical analyses were performed using SAS software. Data on safety and efficacy were evaluated twice yearly by an independent Data Safety Monitoring Board, with predefined stopping rules.

RESULTS

— Screening began on 15 February 1994, and the first subject in this protocol was randomly assigned on 10 September 1996. The actual enrollment period was 6.1 years. By the time randomization was completed (31 October 2002), screening samples for ICA had been obtained from 103,391 relatives. Of these, 97,634 were eligible for further study. Ineligible samples came from individuals without an identified relative with diabetes or not in the age range defined by the protocol. By the end of enrollment, 97,273 samples were analyzed for ICA and 3,483 (3.58%) relatives were ICA positive. Of these, 458 (13.1% of ICA$^+$ individuals) were excluded before randomization because they already had diabetes. A total of 2,523 (72.4% of ICA$^+$ individuals) underwent staging. There were 1,844 relatives with intravenous glucose tolerance FPIR above threshold. As staging continued, a total of 388 relatives were classified as intermediate risk and eligible for randomization; of these, 372 were randomized (97% of eligible subjects), 186 to each study arm (Fig. 1). Table 1 shows baseline characteristics; there were no statistically significant differences between treatment groups. Online appendix Fig. 1 (available at http://care.diabetesjournals.org) shows the frequency distribution of age at randomization by treatment arm.

Participants were followed for a median of 1,382 days (4.3 years; interquartile range 928–1988). Annual rate of loss to follow-up was 0.2%, less than anticipated in the protocol (10%). Annual noncompliance rate was 3.7% in the oral insulin group and 6.6% in the placebo group, with noncompliance being failure to attend for scheduled tests and/or failure to take study medication.

Final primary end point data were available for 98.4% of subjects randomized. Diabetes was diagnosed in 97 participants—44 in the oral insulin group and 53 in the placebo group. The majority (72%) of participants were asymptomatic at the time of diagnosis and/or were detected by study OGT tests. The proportion of participants who developed diabetes, averaged annually over follow-up, was 0.4% per year in the oral insulin group and 8.2% per year in the placebo group. Cumulative incidence of diabetes

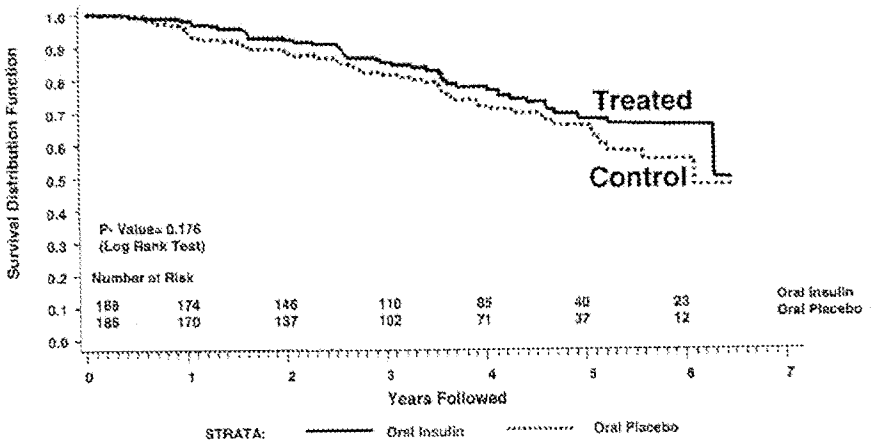

Figure 2—*Kaplan-Meier curves showing the proportion of subjects without diabetes during the trial, by treatment assignment. The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the P values as indicated.*

was similar in both groups (hazard ratio 0.764, 95% CI 0.511–1.142, P = 0.189) (Fig. 2).

Progression to suspected diabetes (diabetes on one occasion not subsequently confirmed) and progression to first abnormal OGT test were examined separately (online appendix Fig. 2*A* and *B*). No treatment differences were found. Time to FPIR below threshold was also examined and again no difference was found (online appendix Fig. 2*C*).

Insulin secretion was examined before diagnosis of diabetes by assessing the C-peptide response during OGT and mixed-meal tolerance tests. There was no difference between groups for peak C-peptide value or area under the curve. Online appendix Fig. 3 shows area under the curve C-peptide values during OGT tests.

There was no difference in glycemia between groups in the intention-to-treat analysis. A secondary regression analysis revealed that, compared with those who did not develop diabetes, subjects who progressed to diabetes had a slight progressive increase in both HbA_{1c} ($P < 0.001$) and area under the curve glucose on serial OGT tests ($P < 0.001$).

There were no serious adverse events and no differences between groups in frequency of adverse events. Rate of chemical hypoglycemia, assessed without ascertainment bias, was 4.4 per 100 patient-years in the oral insulin group and 3.4 per 100 patient-years in the placebo group ($P = 0.287$). There were no reported episodes of severe hypoglycemia.

As noted, the initial entry criterion for IAA was a level ≥80 nU/ml, which was subsequently changed to a level ≥39 nU/ml. There was the suggestion of an increased rate of progression to diabetes in subjects with IAA values ≥80 nU/ml (confirmed on two occasions; $n = 263$) compared with those with IAA values not confirmed ≥80 nU/ml (in which at least one or both measurements were 39–79 nU/ml, $n = 109$, $P = 0.052$) (Fig. 3). Table 2 shows baseline characteristics in those two cohorts; subjects with confirmed IAA values ≥80 nU/ml were younger and more likely to be male and had higher ICA titers, higher frequency of other autoantibodies, and lower levels of C-peptide. All of these characteristics are consistent with higher risk of diabetes.

Among participants with confirmed IAA ≥80 nU/ml ($n = 263$), the proportion who developed diabetes was 6.2% per year in the oral insulin group and 10.4% per year in the placebo group, averaged annually over follow-up (hazard ratio 0.566, 95% CI 0.361–0.888; $P = 0.015$) (Fig. 4). From the data, the delay in diabetes, calculated from median survival times, is projected as 4.5 years. Online appendix Table 1 shows baseline characteristics of this cohort, except for greater proportion of males in the oral insulin group, there were no statistically significant differences between treatment groups.

In contrast, among participants not confirmed as IAA ≥80 nU/ml ($n = 109$), the proportion who developed diabetes was 6.9% per year in the oral insulin group and 2.7% per year in the placebo group, averaged annually over follow-up (hazard ratio 2.702; 95% CI 0.949–7.694; $P = 0.079$; online appendix Fig. 4). Online appendix Table 2 shows baseline characteristics; there were no statistically significant differences between the oral insulin and placebo groups.

In an analysis confined to subjects randomized before the change in IAA criterion on 31 October 1997 ($n = 106$), all of whom had confirmed IAA ≥80 nU/ml, the proportion who developed diabetes was 6.4% per year in the oral insulin

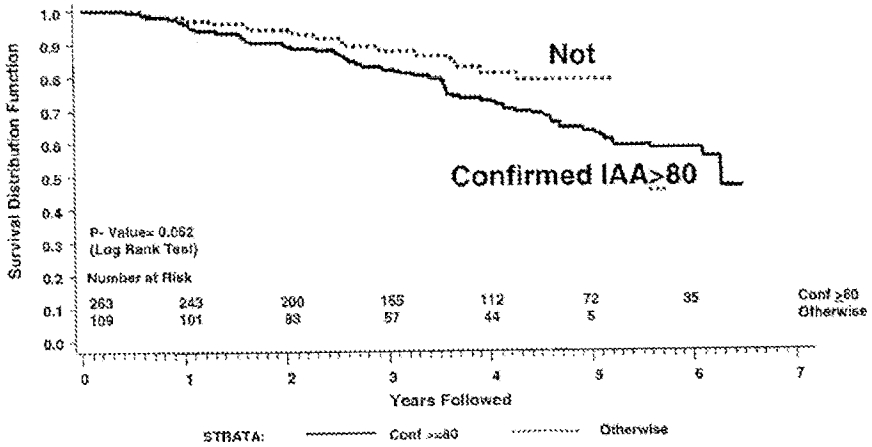

Figure 3—*Kaplan-Meier curves showing the proportion of subjects without diabetes during the trial by baseline IAA level (confirmed value ≥80 nU/ml vs. at least one value 39–79). The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the P values as indicated.*

Table 2—Baseline characteristics of subjects by IAA status

	Not confirmed IAA ≥80	Confirmed IAA ≥80	*P* value
n	109	263	
Median age	13.0 (9–18)	9.0 (6–12)	0.0000
Average FPIR (μU/ml) (SD)	172.1 ± 73.1	155.3 ± 91.4	0.0878
Race			0.9246
White	96 (88.0)	231 (87.8)	
African American	2 (1.8)	5 (1.9)	
Hispanic	7 (6.4)	15 (5.7)	
Other	4 (3.6)	12 (4.5)	
Sex			0.0445
Male	57 (52.2)	167 (63.5)	
Female	52 (47.7)	96 (36.5)	
Relationship to index patient with diabetes			0.1649
Sibling	58 (53.2)	162 (61.6)	
Offspring	31 (28.4)	71 (27.0)	
Parent	9 (8.2)	9 (3.4)	
Second degree	11 (10.0)	21 (7.9)	
Antibody levels			
Median ICAs (JDF units)	40 (20–160)	80 (40–320)	0.0001
Mean IAAs (nU/ml)	72.0 ± 72.3	489.2 ± 547.5	0.0000
GAD antibodies			0.0461
Positive	74 (68.5)	206 (79.3)	
Negative	34 (31.4)	57 (21.6)	
ICA-512 antibodies			0.0043
Positive	44 (40.7)	150 (57.0)	
Negative	64 (59.2)	113 (42.9)	
Micro IAA			0.0000
Positive	4 (5.0)	63 (31.9)	
Negative	76 (95.0)	134 (68.0)	
HbA_{1c} (%)	5.33 ± 0.37	5.35 ± 0.36	0.6112
C-peptide area under curve			
During intravenous glucose tolerance test	40.1 (16.7)	32.8 (15.4)	0.0001
During oral glucose tolerance test	563.9 (225.0)	476.6 (189.1)	0.0002
During mixed-meal tolerance test	443.2 (183.3)	365.2 (169.5)	0.0000

Data are means ± SD, *n* (%), or mean (interquartile range).

group and 11.3% per year in the placebo group, averaged annually over follow-up (hazard ratio 0.539; 95% CI 0.298–0.976; $P = 0.035$) (Fig. 5). From the data, the delay in diabetes, calculated from median survival times, is projected as 4.8 years. Online appendix Table 3 shows baseline characteristics; there were no statistically significant differences by treatment.

CONCLUSIONS — Oral insulin has been used in three studies to test the concept of oral antigen administration in an effort to preserve pancreatic islet β-cell function in newly diagnosed type 1 diabetes (20–22). All three trials failed to show a consistent beneficial effect. Likewise, in BB rats, oral insulin not only failed to prevent type 1 diabetes (23) but, when administered with an adjuvant, actually accelerated the development of diabetes (24). This finding is in stark contrast with the beneficial effects of oral insulin observed in the nonobese diabetic mouse (8–13) and in a transgenic mouse model of virus-induced diabetes (14). Oral antigen administration had only small and inconsistent benefits in clinical trials in multiple sclerosis and rheumatoid arthritis, despite success in animal models of those autoimmune diseases.

Unfortunately, in the primary analysis of relatives selected and randomized in DPT-1, oral insulin did not delay or prevent development of diabetes. There was greater variability in the IAA assay for values 39–79 nU/ml than for values ≥80 nU/ml, particularly in confirmation of a positive result (98.7% overall confirmation for values ≥80 nU/ml compared with 70.6% for values 39–79 nU/ml). This prompted comparison of the rate of evolution of diabetes by entry IAA level (Fig. 2). The cohort with confirmed IAA ≥80 nU/ml (the original entry IAA criterion) progressed to diabetes at a faster rate than those subjects who did not have confirmed IAA ≥80 nU/ml. In addition, those with confirmed IAA ≥80 nU/ml had other risk characteristics that suggest more rapid evolution to diabetes, including younger age, greater likelihood of having other antibodies, and greater loss of β-cell function (lower levels of plasma C-peptide in response to several provocative challenges).

We then examined the effects of intervention in each of these two subgroups. The group with confirmed IAA ≥80 nU/ml showed a beneficial effect of oral insulin, whereas the group who did not have confirmed IAA ≥80 nU/ml showed a trend suggesting a detrimental effect of oral insulin. This group also had a much lower overall rate of development of diabetes. Thus, the significance of this finding is unclear but is reminiscent of the adjuvant induced acceleration of diabetes observed in the BB rat (24).

To gain further insight into the impact of the change that was made in the entry IAA criterion, we performed an analysis confined to subjects randomized before the change in IAA criterion (31 October 1997), all of whom had confirmed IAA ≥80 nU/ml. In this analysis, the results were comparable to those seen in all subjects with confirmed IAA ≥80 nU/ml. There is an obvious lesson for clinical trialists not to tamper with the trial design because enrollment is lagging. One might hypothesize that there might have been a clear beneficial result in the overall trial if the IAA entry criterion had not been changed. However, because none of these subgroup analyses were prespecified, the results suggesting a potential beneficial effect in the subgroup with baseline-confirmed IAA ≥80 nU/ml (either all subjects or those enrolled before the protocol change) can only be deemed hypothesis-generating and not a positive outcome. As a consequence, the successor study group to DPT-1, the Type 1 Diabetes TrialNet clinical trials network, is contemplating a confirmatory study to explore the potential role of oral insulin in delaying or preventing type 1 diabetes in relatives found

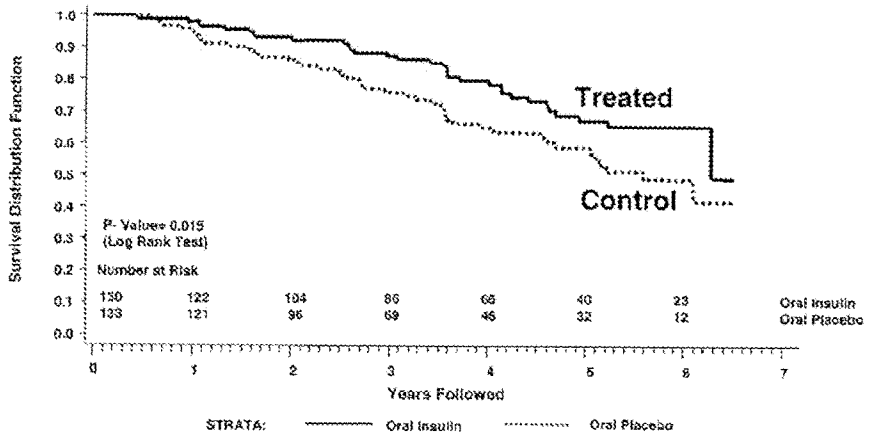

Figure 4—*Kaplan-Meier curves showing the proportion of subjects without diabetes during the trial by treatment assignment for subjects with baseline confirmed IAA ≥80 nU/ml. The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the P values as indicated.*

to be at risk for diabetes with IAA levels similar to those in the DPT-1 subgroup.

There are several possible explanations for failure to demonstrate efficacy in the primary analysis. One is that oral insulin has no effect. Another is that inclusion of subjects with variable and lower risk of diabetes (those with IAA 39–79 nU/ml) may have masked a treatment effect or that in some of these subjects diabetes may have been accelerated. A third possibility is that the dose used in this study was unable to sufficiently stimulate the immune system, but this is difficult to test because we have no established immunologic biomarkers of disease progression. Perhaps if an adjuvant had been used, some effect would have been more evident. In animal models that tested oral insulin, heterologous (either porcine or human) insulin was used. It is possible that homologous insulin, as used here, may have failed to elicit a protective immunologic response. Lastly, the timing of our intervention may have been incorrect. Although there has been speculation that once the immunologic markers used to detect relatives at increased risk for type 1 diabetes are detectable then the destructive immune response may be irreversible by an antigen-based therapy, it is of interest that the subgroup who may have had some benefit of therapy had evidence of being farther along in the disease process (higher antibody levels, greater number of antibodies, and lower levels of C-peptide).

The parameters used to predict development of diabetes in relatives of individuals with diabetes were accurate. Risk was projected to be 26–50%, whereas actual risk was 35% over 5 years. Similarly, in our previously reported parenteral insulin trial, 5-year risk was projected to be >50% and actual risk was 65% (1). The ability to quantify risk in relatives of patients with type 1 diabetes and to randomly assign those relatives in controlled clinical trials permits the design of studies that will ultimately lead to determination of whether the type 1 diabetes disease process can be altered in human beings to delay or prevent the development of clinical diabetes.

Three large randomized controlled trials designed to delay or prevent type 1 diabetes—the two DPT-1 trials and the European Diabetes Nicotinamide Intervention Trial (25)—have failed to demonstrate a treatment effect. It should be noted that of the myriad of interventions that had shown preclinical efficacy, both DPT-1 and the European Diabetes Nicotinamide Intervention Trial chose to use interventions with low toxicity in their attempts to interdict the type 1 diabetes disease process. Thus, it should not be concluded that it is impossible to delay or prevent type 1 diabetes; rather, it may require testing of more potent interventions or combinations of therapies, guided by better understanding of the immunopathogenesis of the disease, to demonstrate attenuation or amelioration of the destructive immune process leading to type 1 diabetes.

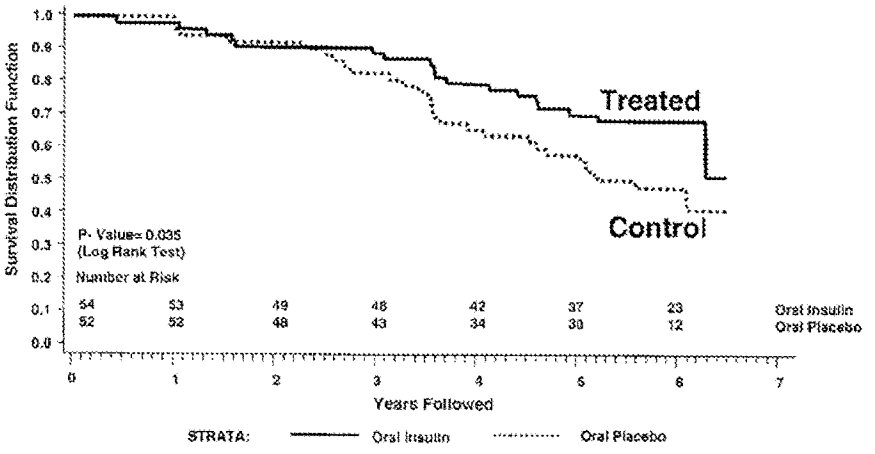

Figure 5—*Kaplan-Meier curves showing the proportion of subjects without diabetes during the trial by treatment assignment for subjects enrolled before protocol change in entry criterion. The number of subjects at risk in each group at each year of follow-up is enumerated at the bottom of the figure. The log-rank test was used for comparison between the groups, with the P values as indicated.*

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George S. Eisenbarth, MD, PhD, a member of the DPT-1 Steering Committee, is an inventor of a patent for the use of oral insulin in inducing immunological tolerance in the prevention or treatment of type 1 diabetes.

Parts of this study were presented at the annual meeting of the ADA, New Orleans, Louisiana, 13–17 June 2003, and at the Immunology of Diabetes Society meeting, Cambridge, U.K., 28–31 March 2004.

APPENDIX

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A complete listing of the DPT-1 Study Group appears in the online appendix. The DPT-1 Protocol and the DPT-1 Manual of Operations are available from the DPT-1 Operations Coordinating Center, University of Miami, 1450 NW 10th Ave., Suite 3054, Miami, FL 33136.

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EXHIBIT K

Oral tolerance and inflammatory bowel disease

Thomas A. Kraus[a] and Lloyd Mayer[b]

Purpose of review

Oral tolerance refers to the ability of the mucosal immune system to actively inhibit systemic immune responses to fed antigens. Recently, clinical trials have used oral tolerance as a therapy for certain chronic inflammatory and autoimmune diseases such as multiple sclerosis and type I diabetes. Inflammatory bowel disease is now widely thought to be caused by the breakdown of oral tolerance through a combination of genetic and environmental factors. Therefore, it seems incongruous that clinicians would try to use oral tolerance therapy to alleviate the symptoms of inflammatory bowel disease. Yet, armed with the results of select animal models, trials have begun for oral tolerance therapy for Crohn's disease. This review will outline the recent advances in understanding oral tolerance, explore the relation between oral tolerance and inflammatory bowel disease, and comment on the likelihood of successful oral tolerance therapy for inflammatory bowel disease.

Recent findings

The results of an oral tolerance trial in Crohn's disease patients in Israel have shown some promising results, whereas the results of studies of experimentally induced oral tolerance in patients with inflammatory bowel disease from the authors' laboratory have shown that feeding a neoantigen in an attempt to induce oral tolerance is not successful in patients with inflammatory bowel disease.

Summary

The fundamental difference in the mechanisms of oral tolerance in mice and humans requires a more focused effort to understand the human mucosal immune system before oral tolerance therapy for autoimmune and chronic inflammatory disorders reaches its full potential.

Keywords

Crohn's disease, inflammatory bowel disease, oral tolerance

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Abbreviations

IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
KLH	keyhole limpet hemocyanin

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Introduction

Recent studies in both mouse models and human tissues have suggested that inflammatory bowel disease (IBD) is a consequence of the breakdown of normal mucosal tolerance. IBD patients have elevated serum antibodies against dietary antigens [1]. Tolerance to normal flora seems to be broken in IBD patients, which suggests active, not suppressed, immune responses against luminal antigens [2–4]. Therefore, the study of oral tolerance can have a significant clinical impact on IBD management. Although the role of $CD4^+$ T cells in the mechanism of murine oral tolerance has been well defined, the contributions of $CD8^+$ T cells, dendritic cells, and other cell types are currently being elucidated. Furthermore, no understanding of the mechanism of oral tolerance in humans exists. Recent work from Rescigno's [5••] lab has implicated an intestinal epithelial cell-derived cytokine that drives the mucosal dendritic cells to secrete antiinflammatory cytokines, contributing to the state of controlled inflammation that is the hallmark of the normal bowel. This cytokine was reduced or absent in intestinal epithelial cells (IECs) from five of seven Crohn's disease patients. Our laboratory [6,7] has published studies showing the absence of a molecule called gp180 from the epithelial cells of IBD patients and that experimental oral tolerance, in both Crohn's disease patients and ulcerative colitis patients in remission, could not be achieved.

These works highlight the relation between oral tolerance, intestinal homeostasis (controlled inflammation), and IBD and may give further insight into the mechanisms of each.

Oral tolerance

The term 'oral tolerance' refers to the active nonresponse to dietary antigens and commensal enteric bacteria or substances administered orally. Given that the systemic immune system must discriminate between self and non-self, the mucosal immune system must discriminate between potentially harmful pathogens and harmless luminal antigens. It has been suggested that the failure to do this results in food allergies and specific food intolerance such as celiac disease (response against food antigens) and Crohn's disease (response against bacterial flora) [8,9]. The mechanisms of oral tolerance induction have been extensively studied in murine systems. Repeated low-dose feeding results in a T cell–mediated suppression of immune responses. Multiple subtypes of regulatory T cells have been identified that involve the secretion of interleukin-10, interleukin-4, and transforming growth factor-β [10–12]. A single high-dose feeding results in T cell anergy or

deletion [13]. Aside from what is known about experimentally induced oral tolerance, there are many other properties of the gut-associated lymphoid tissue that contribute to intestinal homeostasis and the generally suppressive nature of the mucosal immune system.

Regulatory CD8+ T Cells in oral tolerance and inflammatory bowel disease

Although experimental oral tolerance does not require the presence of $CD8^+$ T cells, other studies show that $CD8^+$ T cells can have a suppressor phenotype [14–18]. We have shown that coculturing $CD8^+$ T cells with IECs gives rise to a population of $CD8^+$ cells with a suppressor cell phenotype [6]. This does not occur in cocultures with IECs of IBD patients. Recently, we have shown that there is an oligoclonal expansion of regulatory $CD8^+$ T cells following coculture with normal IECs and that these cells are deficient in IBD patients [19,20]. Because $CD8^+$ T cells were shown to be unnecessary in experimental oral tolerance in murine systems, this might be a fundamental difference in the mechanism of human and murine oral tolerance [14].

Dendritic cells

Recently, progress has been made in understanding the role of dendritic cells in mucosal tolerance. One of the first suggestions that dendritic cells play a role in tolerance was the use of Flt3 ligand in mouse models of oral tolerance induction [21]. In this model, a concurrent increase in dendritic cell populations in the intestine and experimental oral tolerance was observed. It was later shown that dendritic cells could express tight junction proteins and intercalculate their dendrites between intestinal epithelial cells to sample luminal antigens without perturbing the mucosal barrier [22]. Furthermore, intestinal dendritic cells contain components of commensal bacteria [23•]. Investigators have now identified an IEC-secreted cytokine, thymic stromal lymphopoietin, that matures dendritic cells into 'mucosal dendritic cells' that secrete interleukin-10, not interleukin-12, and help promote T_H2 maturation [5••]. Interestingly, in a small survey of Crohn's disease patients, five of seven patients had undetectable amounts of this cytokine. Although the exact significance of this result is unknown, it does suggest that dendritic cells might play an important role in human oral tolerance and, by inference, IBD.

Pathogen recognition in the gut

Familial patterns of IBD suggest that there is a strong genetic component. In a genome-wide screen, genetic linkage analyses implicated *NOD2* as a predisposing genetic factor in Crohn's disease [24,25]. *NOD2* has been identified as a pathogen recognition molecule; *NOD2* recognizes a specific motif of bacterial peptidoglycan, muramyl dipeptide [26–28]. Interestingly, *NOD2*-deficient mice are susceptible to bacterial infection exclusively by the oral route. It has been recently shown that *NOD2* is required for the expression of a subgroup of intestinal antimicrobial peptides known as cryptdins [29••]. The *NOD2* mutations that are found in Crohn's patients are generally loss-of-function mutations, implying that for some patients with Crohn's disease, at least, the initial defect might be the lack the production of these defensins. Gain-of-function mutations also exist, however; these polymorphisms result in constitutive ligand-independent nuclear factor-κB activity, which results in uncontrolled inflammation.

NOD2 is not the only clue that bacterial recognition might trigger colonic inflammation. The lipopolysaccharide-recognizing toll like receptor-4 has been shown to be overexpressed in the gastrointestinal tract in patients with Crohn's disease and ulcerative colitis and in models of murine colitis [30,31]. The recent findings of these bacterial recognition defects and their association to human disease are further evidence that IBD might be caused by a lack of suppression or tolerance to gut microflora [32••].

Oral tolerance therapy in mouse models of human disease

Aside from understanding the mechanisms involved in mucosal tolerance, the idea that oral tolerance could be used to downregulate unwanted immune responses has been gaining popularity. In 1986, the first study was published showing that feeding soluble collagen to mice could inhibit collagen-induced arthritis [33]. Soon after that, other workers used a similar approach to inhibit other murine disease models such as experimental autoimmune encephalomyelitis, the model for multiple sclerosis, as well as models for rheumatoid arthritis, tissue graft rejection, autoimmune uveitis, and type I diabetes [34–40]. Generally, both prophylactic and therapeutic approaches for disease intervention have been very effective.

In studies of oral tolerance therapy for murine colitis, colonic proteins were fed to mice after trinitrobenzene sulfonic acid-induced colitis [41,42]. In this model, trinitrobenzene sulfonic acid is intrarectally administered to a C57/BL10 or SJL/J mouse [43]. The resulting colitis resembles the transmural lesions seen in Crohn's disease and is attributed to an interleukin-12-induced T_H1 response [44]. One possible significant difference between this model and human IBD is that this mouse strain can be tolerized to fed antigens. As mentioned below, studies from our laboratory suggest that IBD patients cannot become orally tolerized to fed antigens. Therefore, it might be more reasonable to expect an oral tolerance response to fed antigens, even colonic proteins, in these mice than in IBD patients.

Oral tolerance in humans

Armed with the effective use of oral tolerance in mouse models of inflammatory and autoimmune diseases, investigators started similar trials in humans. Unfortunately,

the data on the whole do not show particular efficacy [45,46].

The reasons why oral tolerance therapy in humans is not as successful as in mice are unclear. It is possible that the oral tolerance response in humans, using the doses and feeding schedule used in the trials, is not optimal. The optimal oral tolerance response in humans has not yet been fully explored. The first published study showed that repeated feeding of 50 mg of keyhole limpet hemocyanin (KLH), a neoantigen, resulted in a downregulation of T cell responses to subsequent subcutaneous administration of the antigen [47]. In contrast to murine oral tolerance, B cell responses were not inhibited. This study suggested that although the phenomenon of oral tolerance might be shared between species, the mechanisms of tolerance seem to differ.

Our laboratory has previously shown that epithelial cells from both involved as well as uninvolved mucosa of IBD patients lack a molecule called gp180 that is normally expressed on IECs and trophoblasts and has been found to bind to CD8 and associate with CD1d [7,48]. This complex interacts with the TcR/CD8 coreceptor complex on regulatory T cells [49]. To see whether gp180 is necessary for oral tolerance induction, we performed the human oral tolerance protocol in 16 patients with Crohn's disease and ulcerative colitis and more than 30 normal control individuals [50•]. We first found the lowest dose of KLH required for effective oral tolerance (50 mg) in normal control individuals before conducting our study. We found that whereas all the normal control subjects tolerized, 6 of 8 Crohn's disease patients and 7 of 8 ulcerative colitis patients did not tolerize to KLH. In fact, given that T cell responses increased even after the feeding alone, it seemed that feeding the antigen primed the systemic immune response in the ulcerative colitis patients. We concluded that patients with IBD seem to have defective oral tolerance responses and that this correlates with the lack of gp180 expression.

Both Crohn's disease and ulcerative colitis seem to have genetic determinants, Crohn's disease being more closely associated with genetic factors than ulcerative colitis [51]. Therefore, we also studied the oral tolerance responses of nonaffected family members from multiplex IBD families. In this study, 5 of 14 nonaffected family members also did not tolerize to KLH, suggesting that some of these family members might carry the genetic determinant for the loss of tolerance but did not experience IBD [52]. Either they lack exposure to an environmental factor or they lack another contributing genetic factor for the development of IBD.

These studies suggest that oral tolerance therapy for IBD would not be effective; however, in the preliminary studies to date, the results were more promising than other oral tolerance therapeutic trials. Ilan [53•] headed a study with ENZO Biochem in Israel in which 10 Crohn's disease patients with Crohn's disease activity index (CDAI) scores between 200 and 350 were selected and underwent colonoscopies wherein colonic material underwent biopsy and the specimens were prepared for their own orally fed antigenic preparation. After a 16-week treatment of three feedings a week, a median decrease in the CDAI of 129 after 14 weeks was observed, although the score did rise again after treatment ended. Subsets of patients had a reduction of interferon-γ-secreting peripheral blood T cells specific for the fed colonic preparation, an increase of peripheral natural killer T cells, and increases in serum interleukin-4 and interleukin-10 levels. Further multicenter trials are pending from this company.

Conclusion

The gastrointestinal tract uses a profound system of tolerance and controlled inflammation to limit the response to innocuous dietary or bacteria-derived antigens in the gut. When this complex system breaks down, either by a chemical or pathogenic insult, a genetic disturbance, or both, the resulting immune response leads to colitis in mice or IBD in humans.

In a competent mucosal immune system, oral tolerance has been successfully used in laboratories to prevent or to treat mice with a variety of experimental autoimmune disorders with the hope of someday applying this therapy to human disease; however, there seem to be fundamental differences in the mechanisms underlying mouse and human oral tolerance. These differences might partially explain why experimentally induced tolerance is less effective in humans than in mouse models, and why human trials of oral tolerance therapy for inflammatory and autoimmune disorders are less successful than oral tolerance therapy in mouse models.

Using oral tolerance therapy for IBD is complicated further by the fact that the mucosal immune system is already compromised. Statistical analyses of familial inheritance patterns suggest that IBD is a multigenetic disorder. If this is true, then the same nonfunctional or partially effective mucosal immune system that led to IBD is still defective in terms of an ability to tolerize. Indeed, studies from our laboratory suggest that the same protocol that results in oral tolerance in control individuals leads instead to an activation of the immune system in IBD patients. These results suggest that oral tolerance therapy would be ineffective in IBD patients. It is possible, however, that oral tolerance responses to neoantigens are different from those to self-antigens, commensal bacterially derived antigens, or both. Alternatively, the dose and regimen of feeding could lead to differing results.

With a greater understanding of the mechanisms governing the responses of the human mucosal immune

system and oral tolerance will come greater hope for effective treatment of IBD and other inflammatory and autoimmune disorders.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 738–739).

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EXHIBIT L

BIOMEDICAL RESEARCH

Immunology Uncaged

An immunologist argues that to move beyond mice and galvanize clinical research, his field needs its own version of the Human Genome Project

With a routine blood test, your doctor can ascertain how well your metabolism handles lipids and whether you are vulnerable to heart disease. But don't expect to get a test that reveals whether your immune system is working normally or whether you are at risk for, say, autoimmune diseases. The reason: Researchers still can't define what's normal for the immune system, says Mark Davis, an immunologist at Stanford University in Palo Alto, California. Cardiologists can specify healthy levels of LDL, HDL, and triglycerides, but immunologists can't do the same for cytokines, key chemical messengers that trigger immune cells to mature, divide, attack, or perform other actions.

Researchers' reliance on mice deserves some of the blame for this ignorance, says Davis. No mouse-phobe, he keeps 400 cages of the rodents for studies of how T cells recognize pathogen molecules. But mice, says Davis, make a "lousy model" for the human immune system. The human and mouse lineages diverged some 65 million years ago, and the rodent's immune system has adapted to safeguard a small, short-lived animal that scurries around with its nose in the dirt.

However, nobody has cataloged the differences, and as a result, inconsistencies between human and mouse immunity often leave patients in the lurch, Davis says: "Hundreds of clinical trials have been based on curing mice, but almost none led to clinical treatments." Take the case of myelin basic protein (MBP). Injecting MBP into mice causes a condition similar to multiple sclerosis, which can be prevented by doses of proteins that blunt the immune reaction to MBP. But clinical trials of these protective proteins were stopped because they made some people with multiple sclerosis worse.

Failures like that have spurred Davis to call for immunology to go big science in a very human way. If enough labs combine efforts to analyze the thousands of blood samples drawn in the United States or around the world every day, a so-called Human Immunology Project could quickly amass and scrutinize data from large numbers of healthy and sick people, Davis says. Within 5 to 10 years, he predicts, "we could have the first crude benchmarks of immune function." Davis doesn't know what these benchmarks will be—perhaps the levels of particular cytokines or the abundances of certain types of T cells—but he says researchers will probably settle on five or six variables that reflect overall immune status in people, the equivalents of LDL, HDL, and triglyceride levels.

Davis is far from the first to point out the "mouse" problem in immunology. "Studies on mice are very elegant and beautiful, but they aren't reflecting the needs of the [human] population," says Jacques Banchereau, head of the Baylor Institute for Immunological Research in Dallas, Texas.

Hoping to address this problem, Davis over 2 years ago helped to found Stanford's Human Immune Monitoring Center. HIMC analyzes blood samples mainly from Stanford clinical research labs but also from some biotech and pharmaceutical companies, says Director Holden Maecker. The center can measure levels of 50 cytokines, run microarrays to nail down gene activity, and gauge the abundance of more than 30 varieties of white blood cells using flow cytometry, a technology for counting and sifting cells. The researchers receive their results, but HIMC also stockpiles the data. Within a year or two, says Maecker, the center should have enough measurements from multiple studies to start nailing down what's normal for the immune system.

The U.S. National Heart, Lung and Blood Institute in Bethesda, Maryland, has established a similar facility, the Center for Human Immunology, Autoimmunity and Inflammation. The center, says Director Neal Young, provides access to technology, such as the latest flow-cytometry and gene-sequencing machines, that researchers at the National Institutes of Health (NIH) might not be able to afford or have the expertise to use. Many of these studies—a current one tracks the effects of the H1N1 flu vaccine on 200 NIH employees—will be "just looking," Young says, and will accrue large amounts of basic immune data, which the center plans to make public.

Projects like these are a start, says Davis, but a concerted effort is needed. Large-scale projects are not only cheaper because of economies of scale, but they will also use standard procedures that yield comparable results. Moreover, squeezing the most information from blood samples will require an assortment of experts—from clinicians to bioinformatics virtuosos—who aren't available to every lab.

Banchereau is supportive of Davis's call. So is Ralph Steinman of Rockefeller University in New York City, who suggests that such a project could benefit one of his areas of interest: vaccines. "The truth is that to push vaccine science—say, for HIV or cancer—will require a major effort in human immunology."

Davis's push for more basic research on human immunity has also impressed people who control the scientific purse strings. He's received grants for such work from the Howard Hughes Medical Institute and the Bill and Melinda Gates Foundation. And last year the U.S. National Institute of Allergy and Infectious Diseases announced that it would spend $100 million over five years on "human immune profiling research centers" that will track how our immune system responds to jolts such as vaccination and infection. The first grants are due to be awarded in May.

A human immunology project would require more research like this—and more money. Although Davis hasn't submitted a formal proposal, he suspects that the bill would be in the hundreds of millions of dollars, much less than the $4.3 billion (in today's dollars) that the U.S. government spent on the Human Genome Project. Even some of the biggest fans of Davis's idea wonder, however, if such a project is affordable given the current economic climate. But unless we attempt to understand how our own immune system works, "we won't realize the health benefits of immunology," Davis says. "It's not a sustainable strategy to stay focused on mice."

—MITCH LESLIE

Back to basics. Stanford's Mark Davis wants immunologists to pay more attention to human immunity.

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EXHIBIT M

In *Trans* T Cell Tolerance Diminishes Autoantibody Responses and Exacerbates Experimental Allergic Encephalomyelitis[1]

J. Jeremiah Bell,[2]* Rohit D. Divekar,* Jason S. Ellis,* Jason A. Cascio,* Cara L. Haymaker,* Renu Jain,* Danielle M. Tartar,* Christine M. Hoeman,* John C. Hardaway,* and Habib Zaghouani[3]*†

A number of Ag-specific approaches have been developed that ameliorate experimental allergic encephalomyelitis (EAE), an animal model for the human autoimmune disease multiple sclerosis. Translation to humans, however, remains a consideration, justifying the search for more insight into the mechanism underlying restoration of self-tolerance. Ig-proteolipid protein (PLP) 1 and Ig-myelin oligodendrocyte glycoprotein (MOG) are Ig chimeras carrying the encephalitogenic PLP 139–151 and MOG 35–55 amino acid sequence, respectively. Ig-PLP1 ameliorates EAE in SJL/J ($H\text{-}2^s$) mice while Ig-MOG modulates the disease in C57BL/6 ($H\text{-}2^b$) animals. In this study, we asked whether the chimeras would suppress EAE in F_1 mice expressing both parental MHC alleles and representing a polymorphism with more relevance to human circumstances. The results show that Ig-MOG modulates both PLP1 and MOG peptide-induced EAE in the F_1 mice, whereas Ig-PLP1 counters PLP1 EAE but exacerbates MOG-induced disease. This in *trans* aggravation of MOG EAE by Ig-PLP1 operates through induction of PLP1-specific T cells producing IL-5 that sustained inhibition of MOG-specific Abs leading to exacerbation of EAE. Thus, in *trans* T cell tolerance, which should be operative in polymorphic systems, can aggravate rather than ameliorate autoimmunity. This phenomenon possibly takes place through interference with protective humoral immunity. *The Journal of Immunology*, 2008, 180: 1508–1516.

Antigen-specific therapy has become an attractive approach for the treatment of autoimmune diseases because it specifically targets the responses associated with the pathology of the disease, avoiding major side effects (1, 2). Many approaches have proven effective against experimental allergic encephalomyelitis (EAE),[4] but a very limited number of these have efficiently translated into therapies for human multiple sclerosis (MS) and have had only partial effects (2, 3).

Delivery of Ag on Ig has proven to be promising against T cell-mediated autoimmunity including EAE and type 1 diabetes (4–7). Indeed, it has been shown that Ig-proteolipid protein 1 (PLP1), an Ig-encompassing PLP1 peptide corresponding to amino acid sequence 139–151 of PLP (8), is effective against the relapses of EAE (4). Upon aggregation, however, Ig-PLP1 was able to cross-link FcγRs and induce the production of suppressive cytokines such as IL-10 by APCs (4, 8). Consequently, aggregated (agg) Ig-PLP1 was effective in modulating the initial severe phase of PLP1-induced disease, as well as in suppressing the relapses (4). Additionally, agg Ig-PLP1 was able to reverse EAE induced in SJL/J mice by a CNS homogenate, which contains multiple encephalitogenic determinants and provides a full spectrum of pathological responses (5). The approach was not restricted to Ig-PLP1 because agg Ig-myelin oligodendrocyte glycoprotein (MOG), an Ig-encompassing MOG 35–55 peptide, was also able to induce IL-10 production by APCs, display bystander suppression, and reverse CNS homogenate-induced EAE in C57BL/6 mice (5). These observations suggest that the Ig delivery approach may have potential for use under circumstances involving complex MHC polymorphism and diverse T cell specificities. To test these premises, we sought to examine the therapeutic efficacy of agg Ig-PLP1 and Ig-MOG in a setting with more relevance for human MS. Accordingly, Ig-PLP1 and Ig-MOG were used to treat EAE in F_1 mice bred from crossing SJL/J and C57BL/6 mice, two genetically distinct parental strains in which the chimeras were able to reverse the disease. Because these F_1 mice would have a more complex MHC haplotype than their parents, they might give clues to define regimens that would be beneficial for therapy of human autoimmunity. Here, it is shown that both Ig-PLP1 and Ig-MOG are effective against CNS homogenate-induced EAE in (SJL × B10.PL)F_1 mice. However, (SJL × C57BL/6)F_1 animals were resistant to treatment with either chimera. Interestingly, when Ig-MOG was used to treat peptide-induced EAE in the (SJL × C57BL/6)F_1 mice, it showed efficacy against both PLP1- and MOG-induced EAE. However, Ig-PLP1 was effective at suppressing PLP1-induced disease but exacerbated MOG-induced EAE. Evidence is provided indicating that exacerbation of disease by Ig-PLP1 is sustained by PLP1-reactive lymphocytes. Indeed, upon induction of EAE by MOG peptide and treatment with Ig-PLP1, T cells specific

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[4] Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; PLP, proteolipid protein; agg, aggregated; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; HA, hemagglutinin; SP, spleen; LN, lymph node.

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for PLP1 peptide produced IL-5 cytokine which inhibited the production of MOG-specific Abs leading to exacerbation rather than amelioration of EAE. Thus, T cell tolerance that sustains amelioration of EAE in inbred mice evolved to negate protective humoral immunity in the more polymorphic F_1 mice and aggravates autoimmunity.

Materials and Methods

Animals

SJL/J ($H\text{-}2^s$), C57BL/6 ($H\text{-}2^b$), and B10.PL ($H\text{-}2^u$) mice were purchased from The Jackson Laboratory. F_1 (SJL/J × C57BL/6) and (SJL/J × B10.PL) mice were generated by breeding male SJL/J to female C57BL/6 and B10.PL, respectively. All mice were maintained in our animal care facility for the duration of the experiments. All experimental procedures were performed according to the guidelines of the institutional animal care committee.

Antigens

Peptides. The peptides used in this study were purchased from Metabion and were HPLC purified to >90% purity. PLP1 peptide (HSLGKWLGH PDKF) encompasses amino acid residues 139–151 of PLP and is encephalitogenic in SJL/J mice (9). MOG peptide (MEVGWYRSPFSRVVH LYRNGK), encompassing amino acid residues 35–55 of MOG, is encephalitogenic in C57BL/6 and B10.PL mice (10). PLP2 peptide (NT WTTCQSIAFPSK) comprising amino acid residues 178–191 of PLP is also encephalitogenic in the SJL/J mouse (11). Myelin basic protein 3 (MBP3) peptide (VHFFKNIVTPRTP) corresponds to amino acid residues 87–99 of MBP and is encephalitogenic in the SJL/J mouse (12). PLP-LR (HSLGKLLGRPDKF) is a mutant form of PLP1 in which Trp^{144} and His^{147} were replaced with Leu and Arg, respectively, and serves as an antagonist to PLP1 (13). Influenza virus hemagglutinin (HA) amino acid residues 110–120 peptide (SFERFEIFPKE) was used as a negative control (4).

CNS homogenate. Fifty frozen unstripped rat brains (Pelfreez Biologicals) were homogenized in PBS using a Waring blender and adjusted to 300 mg/ml with PBS (4, 5).

Ig chimeras. Ig-PLP1 chimera, harboring PLP1 peptide within the H chain CDR3, has been shown to be effective against EAE when injected into mice in saline (14). Similarly, Ig-MOG harboring MOG 35–55 peptide (5), Ig-MBP3 carrying MBP87–99 (15), and Ig-PLP2 encompassing PLP178–191 (16), have been shown to function as tolerogens when given to mice without adjuvant (4, 5, 15, 16). Ig-PLP-LR, which incorporates PLP-LR altered peptide, functions as an antagonist for PLP1-specific T cells (14). All Ig chimeras have, like Ig-PLP1, had the peptide of interest inserted within the H chain CDR3 region and were constructed using the genes coding for the IgG2b, κ anti-arsonate Ab 91A3 as described previously (14). In brief, the D segment was deleted from the CDR3 of the H chain V region and replaced with a nucleotide sequence that codes for the peptide using mutagenesis procedures similar to those described for the generation of Ig-PLP1 (14). The resulting 91A3-peptide chimeric IgG2b H chain was cotransfected with the parental 91A3κ chain into the non-Ig-producing SP2/0 myeloma B cell line, and the transfectoma cells producing complete Ig-PLP1 were selected with drugs as described previously (14). Transfection, cloning, sequencing, and purification procedures for Ig-MOG, Ig-MBP3, Ig-PLP2, and Ig-PLP-LR are similar to those used for Ig-PLP1 (5–8, 14).

Aggregation of the Ig chimeras

The chimeras were aggregated by precipitation with 50%-saturated $(NH_4)_2SO_4$ as has been previously described (4). Because all chimeras are derived from the same Ig backbone and thereby comprise identical IgG2b isotypes, their Fc-associated functions will be similar.

Induction and scoring of EAE

Induction of EAE has been described previously (4, 5). Briefly, mice (6–8 wk old) were induced for EAE by s.c. injection in the footpads and at the base of the limbs with a 200-μl IFA/PBS (v/v) solution containing 6 mg of CNS homogenate, 100 μg of PLP1, or 300 μg of MOG, along with 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). Six hours later, the mice were given i.v. either 200 (SJL/J × B10.PL) or 500 (SJL/J × C57BL/6) ng of purified *Bordetella pertussis* toxin (List Biological Laboratories). A second injection of *B. pertussis* toxin was given after 48 h. The mice were then scored daily for clinical signs of EAE as follows: 0, no clinical score; 1, loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb paralysis; and 5, moribund or death.

Treatment of EAE

Standard treatment regimen. Mice were treated three times, 4 days apart with 300 μg of agg Ig chimeras at the first observation of clinical signs as described previously (4, 5). Typically, the treatments were given on days 13, 17, 21 postdisease induction by i.p. injection unless indicated otherwise.

Extended treatment regimen. In some experiments the F_1 (SJL/J × C57BL/6) mice received a prolonged treatment regimen consisting of 300 μg of agg Ig chimeras at the first observation of clinical signs and then every 4 days for the duration of the observation period.

In vivo antagonism of PLP1-specific T cells

In some experiments, antagonism of PLP1-specific T cells was conducted during treatment with agg Ig-PLP1. In this case, the mice were induced for EAE with MOG peptide and treated three times, 4 days apart with a mixture of 300 μg of agg Ig-PLP1 and 300 μg of agg Ig-PLP-LR at the first observation of clinical signs as described above.

In other experiments, antagonism of PLP1-specific T cells was conducted before induction of EAE or treatment with agg Ig-PLP1. Accordingly, the mice were given soluble (nonaggregated) Ig-PLP-LR on days −10, −6, and −3 before induction of EAE with MOG peptide. Soluble Ig-PLP-LR was used instead of agg Ig-PLP-LR because this form has been shown to antagonize PLP1-specific T cells (8, 14) and does not induce the production of IL-10 by APCs which could downregulate unrelated T cells by bystander suppression. When the signs of EAE were apparent, the mice were treated with agg Ig-PLP1 according to the standard regimen.

Detection of IFN-γ and IL-5 by ELISA

ELISA was performed according to BD Pharmingen standard protocol. The capture Abs were as follows: rat anti-mouse IFN-γ, R4-6A2 and rat anti-mouse IL-5, TRFK-5. The biotinylated anti-cytokine Abs were rat anti-mouse IFN-γ, XMG1.2, and rat anti-mouse IL-5, TRFK-4. The OD_{405} was read on a SpectraMAX 190 counter (Molecular Devices) and analyzed using SOFTmax PRO 3.1.1 software. Graded amounts of recombinant mouse IFN-γ and IL-5 (BD Pharmingen) were included for construction of a standard curve. The cytokine concentration in culture supernatants was extrapolated from the linear portion of the standard curve.

Inhibition of IFN-γ production by anti-CD4 Ab

F_1 (SJL/J × C57BL/6) mice were induced for EAE with MOG peptide and treated with agg Ig-PLP1 according to the standard treatment regimen. The spleen (SP) and lymph node (LN) anti-MOG IFN-γ responses were then analyzed 3 days after the final treatment. Accordingly, SP or LN cells (1×10^6 cells/well/100 μl) were stimulated with 30 μg/ml MOG peptide (50 μl/well) and 20 μg/ml (50 μl/well) of anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), or anti-$I\text{-}2D^b$ (clone 28-14-8) Ab. For the combination of anti-CD8 plus anti-$I\text{-}2D^b$, 20 μg/ml of each was added. Rat IgG and mouse IgG were included as controls for Abs in matching doses. Cell cultures were incubated for 24 h at 37°C, after which culture supernatants were transferred to anti-IFN-γ-coated plates and cytokine was detected by ELISA as indicated above.

Detection of MOG-specific Abs by ELISA

For detection of anti-MOG Abs in the serum of MOG/EAE mice treated with agg Ig-PLP1, ELISA was used according to the following protocol. Fifty microliters of 0.1 M bicarbonate buffer containing 7 μg/ml MOG peptide was coated into 96-well plates and incubated overnight at 4°C. The plates were then washed and saturated with PBS-3% BSA. Subsequently, serial dilutions of serum were added and incubated overnight at 4°C. Total anti-MOG Abs were detected using anti-κ and anti-mouse Ig coupled to HRP from the Southern Biotechnology Associates clonotyping kit according to the manufacturer's instructions. Class and subclass isotyping were performed in a similar manner using anti-IgG1, -IgG2a and 2b, -IgG3, -IgM, -IgA obtained from the Southern Biotechnology Associates clonotyping kit.

Statistical analysis

Statistical significances comparing EAE disease curves was analyzed by two-way mixed model ANOVA test using GraphPad Prism software

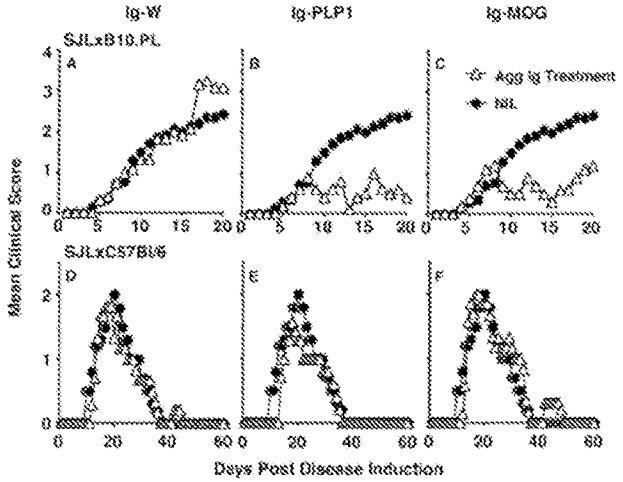

FIGURE 1. Treatment of CNS homogenate-induced EAE in genetically distinct F_1 mice with Ig chimeras shows differential efficacy. Groups of 6- to 8-wk-old (SJL × B10.PL) (*A–C*) or (SJL × C57BL/6) (*D–F*) F_1 mice were induced for EAE by s.c. injections of 6 mg of CNS homogenate along with 200 μg of H37Ra in a 200-μl suspension of PBS/IFA (v/v). When the clinical signs of EAE became evident (loss of tail tone), the mice were then treated i.p. with 300 μg of agg Ig-PLP1 (*B* and *E*), agg Ig-MOG (*C* and *F*), or control agg Ig-W (*A* and *D*) three times at 4-day intervals. The animals were then scored daily for the indicated periods of time.

(GraphPad Software). Significance is indicated as p values in corresponding figure legends with p values <0.05 considered significant and <0.01 highly significant.

Results

Ig-myelin chimeras reverse EAE in (SJL/J × B10.PL) but not (SJL/J × C57BL/6)F_1 mice

Reports indicated that agg Ig-PLP1 (14) and Ig-MOG (5) can reverse CNS homogenate-induced EAE involving diverse T cell specificities (4, 5). The mechanism underlying such effectiveness likely involves cytokine-mediated bystander suppression along with minimal costimulation (4, 5, 8). Because the previous studies were conducted in inbred animals with restricted MHC haplotypes, we sought to test the Ig-myelin system in a more polymorphic animal model, which would be more relevant to human circumstances. As such, we generated two types of F_1 mice, an (SJL/J × B10.PL) and an (SJL/J × C57BL/6)F_1 strain, and tested the agg Ig-myelin chimeras for suppression of EAE. Accordingly, the F_1 female mice were induced for EAE with CNS homogenate, and when the clinical signs of disease became apparent, the animals were given three injections of 300 μg of agg Ig-PLP1 or agg Ig-MOG in saline at 4-day intervals and assessed for reduction in disease severity. Control mice were given agg Ig-W, the parental Ig backbone without any peptide insert. Fig. 1 shows that in the (SJL/J × B10.PL)F_1 mice, those treated with agg Ig-W, similar to untreated animals, had a severe chronic form of EAE, while both agg Ig-PLP1- and agg Ig-MOG-treated animals had mild clinical signs of EAE (Fig. 1, *upper panels*). In contrast, in the (SJL/J × C57BL/6) F_1 mice, while the CNS homogenate injection induced a milder monophasic form of EAE, the treatment with either agg Ig-PLP1, or agg Ig-MOG, had no significant effect in modulating disease (Fig. 1, *lower panels*). These results indicate that the ability of agg Ig-myelin chimeras to treat CNS homogenate-induced EAE depends on the genetic make up of the F_1 mice and, probably, the resulting dominant epitopes.

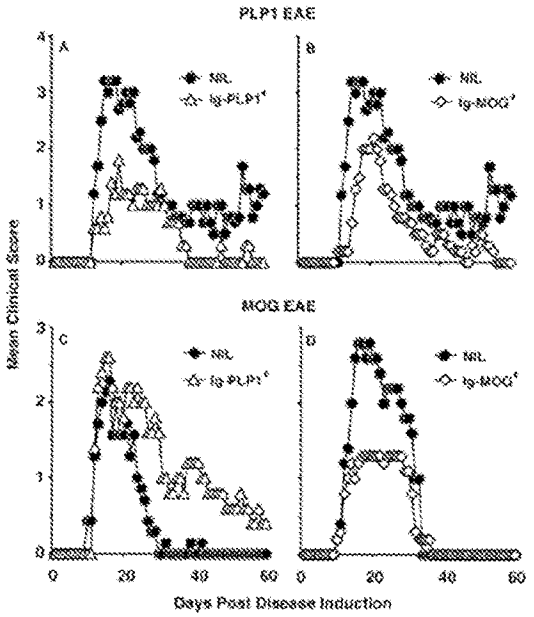

FIGURE 2. Agg Ig-PLP1 treatment exacerbates MOG peptide-induced EAE in SJL × C57BL/6 F_1 mice. Groups (six to eight mice per group) of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were induced for EAE by s.c. injections of 200 μl of PBS/IFA (v/v) containing 200 μg of H37Ra and either (*A* and *B*) 100 μg of PLP1 peptide or (*C* and *D*) 300 μg of MOG peptide. The mice were treated three times every 4 days with 300 μg of either agg Ig-PLP1 (*A* and *C*) or agg Ig-MOG (*B* and *D*) beginning at the first observation of clinical symptoms (day 13) and scored daily for the indicated periods of time. Groups of mice were left untreated (NIL) for comparison purposes. *, Values of $p < 0.05$ as determined by two-way ANOVA described in *Materials and Methods*.

F_1 (SJL/J × C57BL/6) mice induced for EAE with MOG peptide exacerbate their disease when treated with agg Ig-PLP1 but not agg Ig-MOG

Because both agg Ig treatments were able to suppress CNS homogenate-induced EAE in the (SJL/J × B10.PL) but not in the (SJL × C57BL/6)F_1 mice, we sought to determine whether similar effects would occur when the disease is induced by a single, rather than multiple, determinant(s). Accordingly, (SJL/J × C57BL/6)F_1 mice were induced for EAE with either PLP1 (SJL/J-restricted) or MOG (C57BL/6-restricted) peptide and each disease (designated PLP1/EAE and MOG/EAE, respectively) was treated with either agg Ig-PLP1 or agg Ig-MOG. As can be seen in Fig. 2, animals with ongoing PLP1/EAE reduced the severity of their disease when treated with either agg Ig-PLP1 or agg Ig-MOG (*upper panels*). In fact, untreated mice with ongoing PLP1/EAE showed a mean maximal disease severity score of 3.2 ± 0.8 and did not recover during the 60-day monitoring period (Table I). However, mice which were treated with agg Ig-PLP1 recovered by day 37 postdisease induction and exhibited a mean maximal clinical severity of 1.8 ± 0.4. Also, the agg Ig-MOG-treated animals recovered by day 33 with a mean maximal disease severity score of 2.2 ± 0.8. In contrast, mice induced for EAE with MOG peptide showed a significant reduction in disease severity when treated with agg Ig-MOG, but those treated with agg Ig-PLP1 had a pronounced exacerbation of the disease relative to untreated control animals (*lower panels*). The untreated animals with ongoing MOG/EAE had a mean maximal severity of 2.3 ± 0.5 and recovered by day 27. When the disease was treated with agg Ig-MOG, the animals showed a mean maximal disease severity of 1.3 ± 0.5

Table I. *Treatment with agg Ig-PLP1 exacerbates MOG-induced EAE*[a]

Disease Inducer	Treatment with	Day of Disease Onset[b]	Mean Maximal Disease Severity	Day of Recovery[c]
PLP1	Nil	12.0 ± 0.0	3.2 ± 0.8	>60
	agg Ig-PLP1	13.2 ± 2.2	1.8 ± 0.4	37
	agg Ig-MOG	15.3 ± 2.6	2.2 ± 0.8	33
MOG	Nil	11.1 ± 1.0	3.3 ± 0.5	27
	agg Ig-PLP1	12.4 ± 0.5	2.6 ± 0.5	>60
	agg Ig-MOG	11.7 ± 1.0	1.3 ± 0.5	32

[a] The data illustrated in this table were obtained from the mice described in the legend to Fig. 2. These animals were induced for EAE with either PLP1 or MOG peptide and treated with agg Ig-PLP1 or agg Ig-MOG according to the standard regimen.

[b] The onset of EAE represents the day a mouse receives a score of 1 or above, and the indicated numbers represent the mean ± SD.

[c] Mice were considered recovered when their mean clinical score was <0.5 for >3 consecutive days.

and recovered by day 32. To the contrary, mice which were treated with agg Ig-PLP1 showed exacerbated MOG/EAE with a mean disease score of 2.6 ± 0.5 and the mice did not recover for the entire 60-day monitoring period. These results indicate that agg Ig-MOG is effective at treating both PLP1- and MOG-induced EAE, but that agg Ig-PLP1, while effective at suppressing PLP1/EAE, exacerbates MOG-induced EAE and significantly delays spontaneous recovery.

To ensure that exacerbation of disease is not due to spreading to PLP1 epitope after completion of the treatment, the regimen was extended for a longer period of time and the animals were monitored for reversal of disease. As can be seen in Fig. 3, when compared with a group that was given only the standard three-injection regimen of agg Ig-PLP1, the mice that received the extended treatment regimen showed no reduction in disease severity. In fact, the mean maximal disease severity score was 2.4 ± 0.5 which is similar to the 2.5 ± 0.6 score of mice recipient of the standard regimen. Furthermore, both treatment regimens exacerbated the disease and resolution was delayed significantly. Indeed, whereas the untreated (NIL) group showed recovery by day 30 postdisease induction, those treated with the standard or extended agg Ig-PLP1 treatment regimen did not recover by completion of the monitoring period and had a residual mean disease score of 1.5 ± 0.6 and 2.0 ± 0.0, respectively. These results suggest that exacerbation of EAE upon treatment with agg Ig-PLP1 is not due to spreading to PLP1-specific T cells after completion of the short treatment regimen.

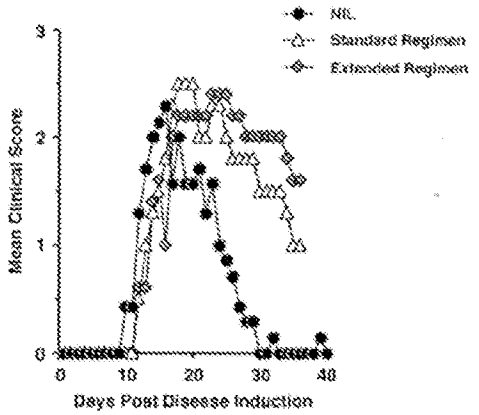

FIGURE 3. Exacerbation of MOG-induced EAE persists even with a continuous agg Ig-PLP1 treatment regimen. Groups (six to eight mice per group) of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were given 200 μl of PBS/IFA (v/v) s.c. injections containing 300 μg of MOG peptide and 200 μg of H37Ra. At the onset of disease (loss of tail tone), a group of mice was given agg Ig-PLP1 three times at 4 days interval (standard treatment regimen), another group was given agg Ig-PLP1 every 4 days for the duration of the experiment (extended treatment regimen) and a third group was left untreated (NIL) to serve as control. The mice were then scored daily for the indicated period of time.

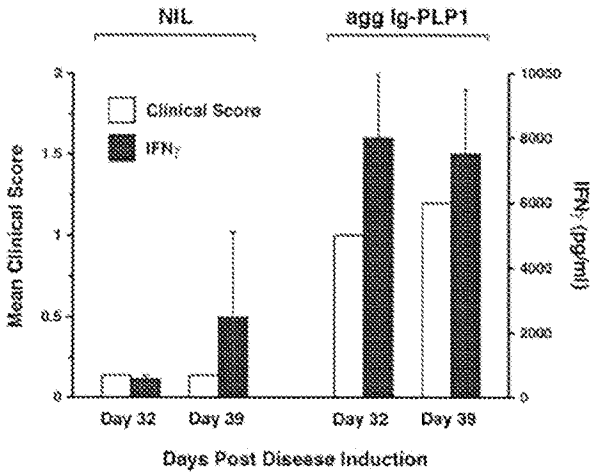

FIGURE 4. agg Ig-PLP1 treatment prolongs MOG-specific IFN-γ production. Groups of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were induced for EAE by s.c. injection of 200 μl of PBS/IFA (v/v) containing 300 μg of MOG peptide and 200 μg of H37Ra. A group of mice was treated three times every 4 days with agg Ig-PLP1 (agg Ig-PLP1) beginning on the day when clinical signs of disease were first observed. An untreated group (NIL) was included for control purposes. The mice were then sacrificed at day 32 or 39 postdisease induction and their LN IFN-γ responses to MOG peptide were analyzed by ELISA. Each filled bar (■) represents the mean ± SD IFN-γ production from six to eight mice. Each open bar (□) represents the mean clinical score of the corresponding group of mice.

agg Ig-PLP1 treatment of MOG-induced EAE sustains IFN-γ production from MOG-reactive cells

To investigate the effects of agg Ig-PLP1 treatment on the MOG-specific T cells, we chose to examine Ag-specific IFN-γ production throughout the course of MOG/EAE with and without agg Ig-PLP1 treatment. Accordingly, (SJL × C57BL/6)F_1 mice were induced for EAE with MOG peptide and treated with agg Ig-PLP1 with the standard (three injections) treatment regimen. On day 32, a point at which the disease resolves in the untreated mice but remains severe in the treated animals, the MOG T cell response was analyzed by measuring MOG-specific IFN-γ production. Interestingly, as shown in Fig. 4, IFN-γ production mirrored the clinical severity of disease in both the treated and untreated mice. Indeed, MOG-specific IFN-γ is clearly produced at higher levels in the LN of agg Ig-PLP1-treated mice as compared with the untreated animals. This is at a time during the disease course in which the treated mice are unable to resolve their disease, and the mean clinical score is significantly higher than in the NIL group. Similar results were observed on day 39 where disease resolution in the untreated mice is maintained, while signs of paralysis remain significant in the treated animals (Fig. 4). Again, IFN-γ production parallels with disease status and was minimal for the NIL group but highly significant in the treated mice. Therefore, it appears that MOG-specific T cells remain active during exacerbation of disease during treatment with agg Ig-PLP1.

Administration of agg Ig-PLP1 has been previously shown to suppress EAE at least partially through the induction of IL-10 production by APCs (4, 5). Because IL-10 can serve as a growth

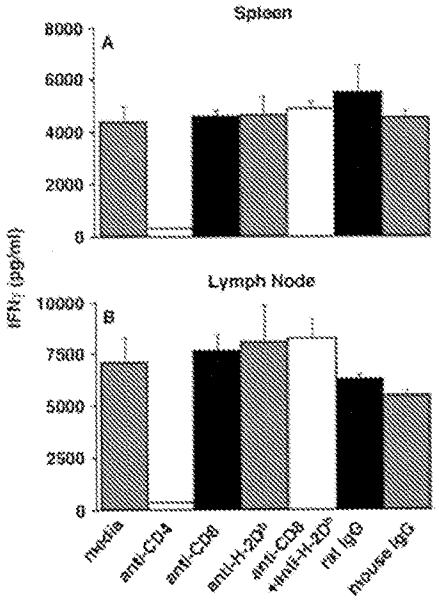

FIGURE 5. MOG-specific CD4, not CD8, T cells mediate the anti-MOG response upon agg Ig-PLP1 treatment of MOG-induced EAE. Groups of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were induced for EAE with MOG peptide as in Fig. 3. The mice were treated three times every 4 days beginning at the onset of clinical signs with 300 μg of agg Ig-PLP1. An untreated control group was included for comparison. Three days after the final treatment, the mice were sacrificed and their (*A*) SP and (*B*) draining LN cells were stimulated with MOG peptide in the presence or absence of blocking anti-CD4, -CD8, or -H-2D^b Abs. The SP and LN cells were used at 1×10^6 cells/100 μl/well. The stimulation used 30 μg/ml MOG peptide and 20 μg/ml anti-CD4, -CD8, or -H-2D^b (anti-class I) Ab. Combination of Abs used 20 μg/ml each. Rat and mouse IgG were included as controls for Abs in matching doses. Each bar represents the mean ± SD of triplicate wells. The results are representative of two independent experiments.

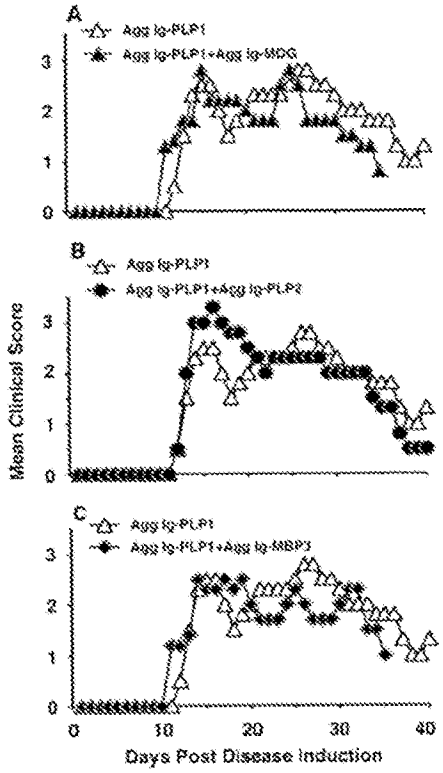

FIGURE 6. agg Ig-PLP1 treatment continues to exacerbate MOG-induced EAE when administered with Igs carrying other myelin epitopes. Groups of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were induced for EAE with 300 μg of MOG peptide as in Fig. 3. At the onset of clinical signs (day 13), the mice were given injections of (*A*) agg Ig-PLP1 + agg Ig-MOG, (*B*) agg Ig-PLP1 + agg Ig-PLP2, or (*C*) agg Ig-PLP1 + agg Ig-MBP3. Each mixture contained 300 μg of agg Ig-PLP1 and 300 μg of the additional chimera. A group of mice treated with 300 μg of agg Ig-PLP1 alone was included for control purposes.

factor for CD8 T cells (17) and it was recently reported that MOG 35–55 encompasses a CD8 epitope (18, 19), we wanted to test whether the IL-10 produced by the APCs upon agg Ig-PLP1 administration was serving to help expand MOG-specific CD8 T cells to exacerbate the disease. To this end, (SJL × C57BL/6)F_1 mice were induced for EAE with MOG peptide and treated three times with agg Ig-PLP1 according to the standard treatment regimen beginning at the first clinical signs of disease. Three days following the final treatment (day 24), the mice were sacrificed and their SP and LN IFN-γ responses to MOG peptide stimulation in the presence of anti-CD4, -CD8, or -H-2D^b Abs were measured by ELISA. As can be seen in Fig. 5, and like the results shown in Fig. 4, there is still a significant IFN-γ production in both the SP and LN of the treated mice (medium). At this time point, the response is comparable to that from mice that had received no treatment when examined at the same time point (data not shown), indicating that agg Ig-PLP1 administration, although able to prolong the MOG response, does not increase the magnitude of the MOG-specific T cell response. When anti-CD4 Ab was added to the cultures, the responses in both the SP (*upper panel*) and LN (*lower panel*) were significantly reduced, indicating that CD4 T cells are responsible, at least in part, for mediating MOG responses. When anti-CD8 Ab or anti-class I (H-2D^b) Ab were added, no change was seen when compared with that of the normal response (medium). Taken together, these results indicate that treatment with agg Ig-PLP1 sustains activation of MOG-specific CD4, not CD8, T cells.

Spreading to epitopes other than PLP1 and MOG is not responsible for exacerbation of MOG-induced EAE by treatment with agg Ig-PLP1

Exacerbation of EAE by agg Ig-PLP1 may have resulted from activation of unrelated T cells as a consequence of a pattern of epitope spreading (20–22) dictated by the F_1 MHC polymorphism. To address this issue, we sought to induce EAE with MOG peptide, treat with agg Ig-PLP1 along with other chimeras carrying different epitopes, and monitor for reduction in disease severity. Three treatment regimens were tested which include agg Ig-PLP1 in combination with agg Ig-MOG; agg Ig-PLP1 along with agg Ig-PLP2 (16), a chimera carrying the I-A^s-restricted PLP2 peptide corresponding to amino acid residues 179–191 of PLP; and agg Ig-PLP1 combined with agg Ig-MBP3 (15) a chimera carrying the promiscuous amino acid sequence 87–99 of MBP. These regimens were applied in the form of a mixture of equal amounts of chimera (300 μg each) according to the standard three injections at 4-day intervals. A group of mice recipient of agg Ig-PLP1 alone was included to serve as a control. As can be seen in Fig. 6, none of the combinations were able to induce reduction in the severity or duration of the disease. Indeed, the mean maximal disease score was 2.8 ± 0.8 for treatment with agg Ig-PLP1 plus agg Ig-MOG; 3.3 ± 1.0 for agg Ig-PLP1 plus agg Ig-PLP2; and 2.5 ± 1.0 for the treatment with agg Ig-PLP1 plus agg Ig-MBP3. This is similar to the 2.8 ± 1.0 score observed during treatment with agg Ig-PLP1

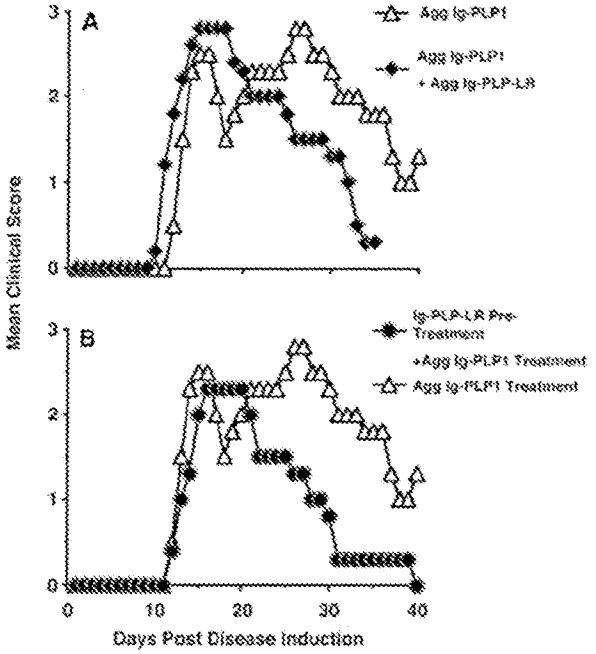

FIGURE 7. Antagonism of PLP1-specific T cells by Ig-PLP-LR nullifies agg Ig-PLP1- mediated exacerbation of MOG-induced EAE. *A*, Groups of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were induced for EAE with 300 μg of MOG peptide and treated three times every 4 days beginning at the onset of clinical signs with either 300 μg of agg Ig-PLP1 alone or in combination with 300 μg of agg Ig-PLP-LR antagonist. The mice were monitored daily and assessed for clinical scores throughout the observation period. *B*, Five- to 7-wk-old (SJL × C57BL/6)F_1 mice were given 300 μg of soluble Ig-PLP-LR i.p. at days −10, −6, and −3 before disease induction. These mice along with a group of 6- to 8-wk-old untreated (SJL × C57BL/6)F_1 mice were then induced for EAE with 300 μg of MOG peptide. At the onset of clinical symptoms, both groups of mice were treated three times, 4 days apart with 300 μg of agg Ig-PLP1 and monitored for clinical signs of EAE daily until day 40 post disease induction.

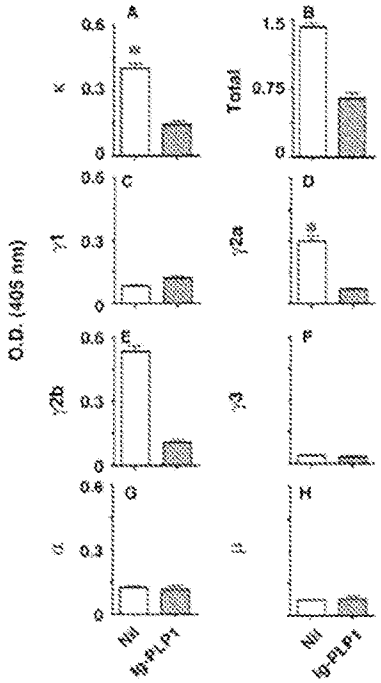

FIGURE 8. agg Ig-PLP1 treatment diminishes anti-MOG Abs. Groups of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were induced for EAE with 300 μg of MOG peptide and treated with 300 μg of agg Ig-PLP1 three times at 4-day intervals beginning at the onset (loss of tail tone) of clinical signs of EAE. The mice were bled via tail veins 4 days after the last agg Ig-PLP1 injection. A group of mice induced for MOG/EAE but treated with PBS instead of agg Ig-PLP1 was included for comparison purposes. κ-bearing (*A*) and total (*B*) MOG Abs from the untreated (Nil) and agg Ig-PLP1-treated (Ig-PLP1) mice were measured by ELISA using the Southern Biotechnology Associates kit as described in *Materials and Methods*. Isotyping for γ1 (*C*), γ2a (*D*), γ2b (*E*), γ3 (*F*), α (*G*), and μ (*H*) class and subclass of anti-MOG Ab were also determined by ELISA using the Southern Biotechnology Associates kit as described in *Materials and Methods*. Serial serum dilutions were used in these analyses and the results shown were those obtained with 1/500 dilution. Each bar represents the mean ± SD of triplicates from five mice. *, Significant difference relative to control untreated mice as analyzed by Student *t* test.

alone. Moreover, the patterns of clinical signs were similar throughout the duration of the monitoring time period. Thus, the exacerbation of MOG/EAE by agg Ig-PLP1 is not due to spreading to other epitopes such as PLP2 or MBP3. Interestingly, even agg Ig-MOG when combined with agg Ig-PLP1 is no longer able to modulate the disease.

Antagonism of PLP1-specific cells by Ig-PLP-LR modulates agg Ig-PLP1-mediated disease exacerbation

Epitope spreading does not seem to be responsible for exacerbation of MOG-induced EAE by agg Ig-PLP1. Treatment with Ig-MOG combined with Ig-PLP1 does not modulate the disease possibly indicating that MOG-specific T cells may not be responsible for exacerbation of disease. Thus, it may be that agg Ig-PLP1 in the context of $H\text{-}2^{s\ /\ b}$ stimulates rather than inactivates PLP1-specific T cells to sustain the severity of EAE. To test this premise, we sought to antagonize PLP1-specific T cells and determine whether disease exacerbation by agg Ig-PLP1 persists.

It has previously been shown that replacement of the TCR contact residues 144W and 147H with 144L and 147R of PLP1 generates a peptide designated PLP-LR that functions as an antagonist of PLP1-specific T cells (13). Also, PLP-LR was previously expressed on Ig and the resulting Ig-PLP-LR chimera antagonized PLP1-specific T cells effectively (8, 14). Herein, Ig-PLP-LR was used to antagonize PLP1-specific T cells to test for the effect of agg Ig-PLP1 on MOG-induced EAE. Accordingly, (SJL × C57BL/6)F_1 mice were induced for EAE with MOG peptide and when the clinical signs of disease became apparent, the mice were given agg Ig-PLP1 alone or in combination with agg Ig-PLP-LR and monitored daily for reduction in disease severity. As illustrated in Fig. 7*A*, while the mean maximal score remains unchanged in the two groups (2.8 ± 0.8 vs 2.8 ± 1.0), the mice treated with the agg Ig-PLP1 and agg Ig-PLP-LR combination regimen reduced the severity of their paralysis earlier and resolved their disease by day 35 following disease induction. To ensure that exacerbation of disease involves PLP1-specific T cells, we used soluble Ig-PLP-LR to antagonize the cells before disease induction with MOG and treated with agg Ig-PLP1 alone. The choice of soluble Ig-PLP-LR for T cell antagonism before disease induction stems from the observation that this form is effective for prevention of disease and circumvents IL-10 bystander suppression as soluble chimeras do not induce IL-10 production by APCs (4). As indicated in Fig. 7*B* when Ig-PLP-LR was given before disease induction the mean clinical score was slightly lower relative to mice not given Ig-PLP-LR (2.8 ± 0.5 vs 2.3 ± 0.5). However, the severity

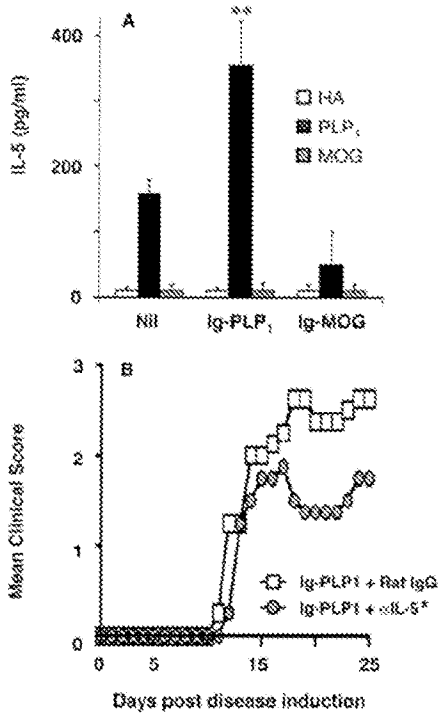

FIGURE 9. agg Ig-PLP1 treatment exacerbates MOG/EAE through induction of IL-5 production by PLP1-specific T cells. In *A*, groups of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were induced for EAE with 300 μg of MOG peptide and treated with 300 μg of either agg Ig-PLP1 or agg Ig-MOG three times at 4-day intervals beginning at the onset (loss of tail tone) of clinical signs of EAE. A group of mice that was induced for MOG/EAE but was not treated with any chimera (Nil) was included as control. The mice were sacrificed 4 days after the last treatment with the chimeras and their LN cells (5×10^5 cells/well) were stimulated in vitro with PLP1 (15 μg/ml), MOG (30 μg/ml), or HA (30 μg/ml) and cytokine production was measured by ELISA as described in *Materials and Methods*. Each bar represents the mean ± SD of triplicate wells. In *B*, groups of MOG/EAE mice were treated with agg Ig-PLP1 as in *A* and received an injection of 500 μg of anti-IL-5 Ab (TRFK-5) or rat IgG control along with the second agg Ig-PLP1 treatment. The mice were then monitored daily for clinical signs of EAE. Each point represents the mean score of eight mice. *, Values of $p < 0.05$ as determined by two-way ANOVA described in *Materials and Methods*.

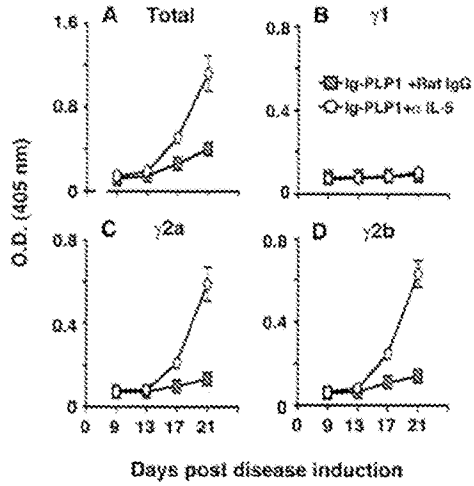

FIGURE 10. Neutralization of IL-5 during treatment of MOG/EAE with agg Ig-PLP1 restores production of MOG-specific Abs. Groups of 6- to 8-wk-old (SJL × C57BL/6)F_1 mice were induced for EAE with 300 μg of MOG peptide and treated with 300 μg of agg Ig-PLP1 three times at 4-day intervals beginning at the onset (loss of tail tone) of clinical signs of EAE. An injection of 500 μg of anti-IL-5 Ab (TRFK-5) (circles) or Rat IgG control (rectangles) was also given along with the second agg Ig-PLP1 treatment (day 13). The mice were bled via tail veins before the treatment with agg Ig-PLP1 (day 9) and once every 4 days thereafter. Total (*A*) and γ1 (*B*), γ2a (*C*), and γ2b (*D*) isotypes of anti-MOG Abs were determined by ELISA as described in Fig. 8. Serial serum dilutions were used in these analyses and the results shown were those obtained with 1/500 dilution. Each point represents the mean ± SD of triplicates from five mice.

of clinical signs declined significantly earlier and the disease resolved by day 30 while those not pretreated with Ig-PLP-LR did not recover for the 40-day disease monitoring period. Altogether, these results indicate that PLP1-specific T cells need to be functional to contribute to disease exacerbation upon treatment of MOG-induced EAE with agg Ig-PLP1.

Treatment of MOG EAE with agg Ig-PLP1 induces IL-5 that inhibits anti-MOG Ab response and sustains exacerbation of disease in F_1 mice

The results presented above indicate that MOG-specific T cells are activated during treatment with agg Ig-PLP1 but not required for exacerbation of disease while PLP1-specific T cells are. The question then is how PLP1-specific T cells contribute to exacerbation of disease.

MOG EAE usually involves Abs that contribute to the pathology of EAE (23, 24). If treatment with agg Ig-PLP1 leads to an increase in the production of MOG-specific Abs, it would result in exacerbation of disease. To address this postulate, F_1 mice were immunized with MOG peptide, treated with agg Ig-PLP1 and their sera were tested for the presence of anti-MOG Abs. The results illustrated in Fig. 8 show, surprisingly, a significant reduction rather than increase in *n* bearing (Fig. 8*A*), as well as total, anti-MOG Abs (Fig. 8*B*). Furthermore, the inhibition is more prevalent for both IgG2a and IgG2b isotypes (Fig. 8, *D* and *E*) rather than IgG1, IgG3, IgA, IgM classes of Ig (Fig. 8, *C* and *F–H*).

Because PLP1-specific T cells seem to be involved in agg Ig-PLP1-mediated exacerbation of MOG EAE and cytokines can control the magnitude and class of Ab responses, we sought to determine whether PLP1-specific cells and their cytokines interfere with the production of anti-MOG Abs. In an initial experiment, we immunized F_1 mice with MOG peptide, treated the animals with agg Ig-PLP1 or control Ig-MOG that did not exacerbate the disease, and tested the T cells for differential cytokine expression. Among many cytokines tested including IFN-γ, IL-4, TGF-β, IL-17, only IL-5 was differentially produced in agg Ig-PLP1- vs agg Ig-MOG-treated mice (Fig. 9). Indeed, IL-5 is significantly increased upon stimulation of LN cells with PLP1 but not MOG peptide in the agg Ig-PLP1-treated mice (Fig. 9*A*). However, in the mice treated with agg Ig-MOG, neither PLP1 nor MOG peptide stimulation was able to induce significant IL-5 production. In fact, IL-5 in these mice was at background level relative to mice that were not treated with any chimeras. These results suggest that IL-5 produced by PLP1-specific T cells may be responsible for disease exacerbation in agg Ig-PLP1-treated MOG EAE. To test this premise, F_1 mice were induced for EAE with MOG peptide, treated with agg Ig-PLP1 with either anti-IL-5 Ab or rat IgG control, and the severity of disease was monitored. Fig. 9*B* shows indeed that neutralization of IL-5 in vivo inhibits disease exacerbation and the maximal severity of paralysis was reduced from 2.3 ± 0.2 to 1.3 ± 0.4. These results indicate that IL-5 from PLP-1-specific T cells is responsible for disease exacerbation in agg

Ig-PLP1-treated animals. Because the mice with exacerbated disease had significant IL-5, displayed diminished production of MOG Abs, and neutralization of IL-5 reduced disease severity, we suspected that IL-5 exacerbates disease by suppression of MOG Abs. If this is the case, then neutralization of IL-5 which inhibited disease severity should restore production of MOG Abs. Indeed, Fig. 10 shows that mice recipient of anti-IL-5 Ab during treatment with agg Ig-PLP1 restored production of total anti-MOG Abs (Fig. 10*A*). Moreover, IgG2a and IgG2b isotypes which were reduced by treatment with agg Ig-PLP1 were restored by neutralization of IL-5 (Fig. 10, *C* and *D*) but IgG1 subclass which was not affected by treatment with Ig-PLP1 was not increased by neutralization of IL-5 (Fig. 10*B*). These results indicate that IL-5 from PLP1-specific T cells diminishes MOG Abs and sustains exacerbation of MOG EAE in F_1 mice.

Discussion

In previous studies, we showed that both agg Ig-PLP1 and agg Ig-MOG effectively suppress EAE by a "dual modal" mechanism involving minimal costimulation and IL-10 bystander suppression (4, 5, 8). The approach is attractive because it targets both Ag-specific T cells, as well as nearby autoreactive T cells of unrelated specificities (4, 5, 8). These reports examined the efficacy of the Ig delivery system for treatment of autoimmunity in nonpolymorphic inbred mouse strains. However, these animals do not nearly represent the genetic diversity seen in humans. In this study, we examined whether the Ig approach would be able to exert similar suppressive effectiveness in a polymorphic setting with relevance to humans. Accordingly, F_1 mice were generated by breeding SJL/J to B10.PL or C57BL/6 and agg Ig-PLP1 as well as agg Ig-MOG were tested for suppression of CNS homogenate-induced EAE where the full spectrum of epitopes restricted to both parental haplotypes is at play and the chimeras would have to modulate diverse T cell specificities to ameliorate the disease. Both agg Ig-PLP1 and agg Ig-MOG were quite effective at reversing EAE induced with CNS homogenate in the (SJL/J × B10.PL)F_1 mice (Fig. 1). Unexpectedly, however, when the same experiment was performed in the (SJL/J × C57BL/6)F_1 strain, neither Ig chimera was able to ameliorate disease. This was intriguing and prompted investigation at the single epitope level where EAE is induced by an epitope restricted to the haplotype of one parent and the treatment is made with a chimera carrying a peptide with the same parental restriction or a peptide restricted to the other parent (in *trans* treatment). Accordingly, groups of (SJL × C57BL/6)F_1 mice were induced for EAE with PLP1 or MOG peptide and the animals were treated with either agg Ig-PLP1 or agg Ig-MOG. It was observed that both chimeras were able to ameliorate PLP1-induced EAE (Fig. 2 and Table I). However, in the animals induced for EAE with MOG peptide, Ig-MOG reduced the severity of disease while in *trans* treatment with Ig-PLP1 exacerbated the clinical signs of MOG/EAE, where the initial phase of disease was more pronounced and the resolution was delayed. Moreover, prolonged in *trans* treatment with agg Ig-PLP1 did not change the disease exacerbation pattern (Fig. 3) suggesting delayed spreading to PLP1 epitope after completion of the three-injection regimen was not the mechanism responsible for disease exacerbation. In fact, MOG-specific IFN-γ production was evident during exacerbation of disease by agg Ig-PLP1 treatment (Fig. 4). Also, spreading to epitopes other than PLP1 and MOG was excluded because when Ig-PLP1 was accompanied with Ig-MBP3 or Ig-PLP2 exacerbation of EAE persisted (Fig. 6). This was intriguing and led us to suspect involvement of CD8 T cells. Indeed, there are reports in the literature indicating that MOG 35–55 encompasses an epitope recognized by CD8 T cells in the C57BL/6 mouse (18, 19). Because aggregation of the Igs induces the APCs to produce IL-10, which is a growth factor for CD8 T cells (17), and we found that the anti-MOG response is maintained in agg Ig-PLP1-treated mice compared with untreated, there was a possibility that agg Ig-PLP1 was sustaining activation and expansion of MOG-specific CD8 T cells. However, this postulate proved incorrect as Ag-specific IFN-γ could be inhibited only in the presence of anti-CD4 Ab indicating that CD8 T cells play little or no role in agg Ig-PLP1-mediated exacerbation of MOG-induced EAE (Fig. 5).

In the face of this dilemma, we were left with the possibility that agg Ig-PLP1 may be activating, rather than tolerizing, PLP1-specific T cells, leading to exacerbation of disease. To examine this possibility, agg Ig-PLP1 was combined in a treatment with Ig-PLP-LR, an Ig carrying a PLP1 antagonist (8). The results indicated that agg Ig-PLP-LR reduced the severity of disease during treatment with agg Ig-PLP1 (Fig. 7). Furthermore, when antagonism was conducted before disease induction with MOG peptide, treatment with agg Ig-PLP1 exacerbation of disease was nullified (Fig. 7). This indicates that PLP1-specific T cells are required for exacerbation of disease (Fig. 7). The question then is how these T cells aggravate MOG/EAE. Cytokine screening analysis indicated that in the MOG/EAE mice treated with agg Ig-PLP1 there was production of IL-5 by PLP1-specific T cells that was not observed in untreated or Ig-MOG-treated mice (Fig. 9). Given that IL-5 is a Th2 cytokine usually associated with modulation of autoimmunity, we concluded that it might be indirectly involved in the exacerbation of disease. Knowing that MOG/EAE involves Abs (23, 24), we suspected that IL-5 may be interfering with such Ab responses to aggravate the disease. Surprisingly, however, treatment with agg Ig-PLP1 reduced MOG Ab responses and more specifically the IgG2a and IgG2b isotypes (Fig. 8). Furthermore, neutralization of endogenous IL-5 modulated the disease (Fig. 9) and restored Ab responses with reincrease of IgG2a and IgG2b anti-MOG isotypes to significant serum levels (Fig. 10). Because IL-5-deficient mice manifest signs of EAE similar to wild-type mice (25), it is possible that other cytokine products of Th2 or T regulatory cells contribute to the Ig-PLP1-mediated aggravation of EAE. The fact that disease progression occurs when serum anti-MOG Ab titers diminish and the severity is reduced when the Ab titer reincreases (by neutralization of IL-5) suggests that the Abs may play a protective role. It has previously been shown that MOG-specific Abs directed against conformational epitopes are pathogenic while those recognizing linear epitopes have no demyelinating effects (26, 27). In this study, because the Abs are induced and detected by the MOG 35–55 peptide, they represent anti-linear epitope Abs that seem to play a protective rather than pathogenic role. Given that MOG is restricted to C57BL/6 haplotype and that this strain is unable to develop pathogenic Abs to conformational MOG epitopes (28), it is likely that the linear epitope-specific Abs contribute protective rather than demyelinating functions. The conclusion that can be drawn from these studies suggests that T cell tolerance in the context of genetic polymorphism could nullify protective humoral responses and aggravate rather than ameliorate autoimmunity.

Overall, Ag-specific therapies are ideally more suitable for treatment of autoimmune disorders than non-Ag-based therapies, presumably because they affect the specific cells responsible for the pathogenesis of the disease. However, complex polymorphisms which could result in unbalanced MHC expression (29) need to be taken into consideration to devise effective Ag-specific therapy against the disease.

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EXHIBIT N

Animal models of human type 1 diabetes

Matthias von Herrath & Gerald T Nepom

Type 1 diabetes is an immune-mediated disease in which pancreatic insulin-producing beta cells are damaged and destroyed. Animal models have served a prominent function in the development of the present ideas of pathogenesis and approaches to therapy. This commentary addresses the utility and limitations of these models for facilitating the 'translation' of immunology research into clinical applications.

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In autoimmune diabetes (also called 'type 1A diabetes' (T1D)), the accumulation of islet antibodies with differential specificities for beta-cell proteins, in combination with genotyping for susceptibility-related alleles, can be used to predict the risk of developing clinical diabetes. Those correlates, along with the presence of T cells specific for islet antigens and partial clinical amelioration after systemic immunosuppression[1–5], indicate an immunological basis for the pathogenesis of T1D, yet knowledge about this disease is incomplete. The therapeutics now available are inadequate, which provides an impetus for the development of new approaches using animal models to better understand the pathogenesis and obtain new treatments. However, clinical 'translation', in this case mostly from mouse to man, has been slow and fraught with difficulties. It is therefore important to take a critical look at the present diabetes mouse models and their utility for creating scenarios that might or might not apply to the pathogenesis of human T1D.

Obstacles to translational approaches

The main issue that makes the rational and targeted application of animal models difficult for T1D is that very little is known about the pathogenesis of the human disease. Which beta-cell antigens initiate human T1D, the extent of heterogeneity between individual cases of T1D, whether viral infection of beta cells is commonly involved and through which mechanism beta cells usually die are all poorly understood. Clinically, T1D occurs with considerable heterogeneity in terms of age at onset, remaining beta-cell mass as assessed by C peptide (a cleavage product generated and measurable in peripheral blood during insulin production) and the severity of complications. Any single animal model is therefore unlikely to address the issue of population heterogeneity and, indeed, animal models have been instructive about many diverse scenarios that potentially could occur in human T1D. For example, many death pathways, including those involving perforin, granzyme, the receptor Fas (CD95) and its ligand, cytolytic $CD8^+$ T cell killing or cytokines, can lead to beta-cell demise. Human T1D should not be expected to involve only one single pathway or cause[6]. Similarly, animal models have allowed probing of the relative importance of cytokine pathways in enhancing or preventing T1D. However, only a few pathways have been shown to be essential, whereas there is considerable redundancy among others. This situation carries important therapeutic implications in that it may be difficult to treat or prevent T1D by blocking only one cytokine or signaling pathway, and combination therapies must be considered.

Overall, the frustration of 'translating' findings from animal models to human therapeutics is illustrated by the failure, when ultimately tested in humans, of several preventive and interventional approaches that have been effective in nonobese diabetic (NOD) mouse models (**Box 1**). There are several reasons, in our opinion, for this failure, and there is substantial room for improvement. First and foremost, various animal models should be investigated in terms of which characteristics of human T1D they most accurately reflect. For linkage of a given model to certain cases of human T1D, specific genetic or biological markers that can be obtained from peripheral blood must be established so the relevant parallels can be drawn. In particular, for the movement of therapies from mouse to man, the identification of biomarkers, especially those that could potentially serve as trial outcomes, will probably be crucial for classifying patients in terms of the correct and most appropriate intervention strategy.

Second, a substantial problem is the lack of biomarkers that can be measured in peripheral blood that would allow prediction of the favorable outcome of a given intervention. These should be assessed and developed in mouse models; however, in most cases, immunological parameters are assessed in lymphoid organs such as the spleen or pancreatic lymph node, which are not accessible in humans, for obvious reasons. This is particularly problematic for the use of beta-cell antigen–specific mucosal immunization (such as oral or nasal insulin) to induce adaptive regulatory T cells that can establish long-term tolerance. Here there is no direct and easy way to 'translate' dose and scale. In addition, the administration of many antigens results in a bell-shaped dose-response curve in which only intermediate amounts have the desired effect of inducing regulatory T cells, whereas too little or too much antigen has no effect in preventing T1D[5,6]. Such issues, coupled with the lack of suitable biomarkers that can be measured in peripheral blood to monitor therapeutic success (in both animal models and humans), make studies to find the correct dose and regimen often lengthy and cumbersome.

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Box 1

Examples of successful therapies in NOD mice that have so far failed in human trials

- Bacille Calmette-Guerin (TNF-inducing tuberculosis vaccination, administered once)
- Nicotinamide
- Oral insulin (TrialNet trial; oral insulin given daily)
- Altered peptide ligands (Neurocrine trial)
- Subcutaneous insulin (whole insulin given subcutaneously at a low dose)
- Intranasal insulin (Finnish trial; insulin given daily)
- Antibody to the interleukin 2 receptor

Examples of successful therapies in NOD mice now being tested in human trials

- Antibody to CD20
- Antibody to CD3 (initial success as described in refs. 1,2)
- Antibody to thymocyte globulin
- Rapamycin plus interleukin 2

Therapies that failed or had no effect in NOD mice, now being tested in human trials

- CTLA-4–immunoglobulin (blockade of costimulation)

Adapted from Ref. 6 with permission.

In the context of these concerns, what present models show promise for filling some of the key translational gaps? Can an inventory of flexible preclinical models be built to facilitate research progress and make clearer recommendations for their use? There are many opportunities uniquely suited for appropriate animal studies (**Box 2**).

Present diabetes models

The NOD mouse strain is the 'workhorse' of the T1D preclinical 'stable'. The diabetes of NOD mice is highly penetrant, rapid in onset and sensitive to immunomodulation. Indeed, over 200 perturbations of the immune environment are known that can prevent or reverse disease in NOD mice[7]. One of the important lessons of this model, however, is the importance of genetic heterogeneity. The NOD strain has many genes related to susceptibility to autoimmunity, which provides a fertile background onto which alternative major histocompatibility complex (MHC) genes confer alternative disease phenotypes. Thus, the NOD H-2^g strain develops thyroiditis but not diabetes[8], whereas the NOD DQ8 strain develops myocarditis, not diabetes[9]. Conversely, the NOD H-2^{g7} MHC allotype is not sufficient to confer diabetes when introduced into a different mouse strain that lacks the other NOD-related autoimmune-susceptibility loci[10]. It is the combination of a specific MHC with a large set of permissive background genes that produces the NOD phenotype—a rather unique set of genotypic circumstances that is unlikely to exist in a substantial fraction of the human population, if it exists at all.

So although a focus on NOD autoimmunity-related genes may generate considerable information about the potential pathways that facilitate diabetes in this model, this approach is not the most direct pathway to an understanding of human disease. Instead, we encourage reversing the conventional mouse genetics approach to identify human susceptibility-related loci. Instead of looking for homologs in humans of the NOD autoimmunity-related genes, variations and homologs of the human diabetes susceptibility-related alleles should be introduced into mouse strains that carry the NOD H-2^{g7} MHC but that lack the NOD background autoimmunity-related genes. An increase in autoimmunity or disease penetrance in this context would pinpoint specific molecular pathways associated with disease progression and simultaneously confirm biomarkers (whether autoantibodies, serum cytokines or other inflammation surrogates) of potential intermediate phenotypes.

Another underused model resource is the set of spontaneous mouse models of T1D or other autoimmune diseases that result from specific known mutations or pathway defects. In this context, it is helpful to think of human autoimmune disease as a set of alternative deleterious circumstances, such as flawed immune tolerance due to defects in lymphocyte selection, and/or deficient activation-induced cell death, and/or the deficient generation of regulatory T cells or function, and/or overexuberant innate activation, and/or dysregulated activation of lymphocytes, monocytes and so on. For successfully understanding and monitoring of these pathways in human patients, accurate biomarkers must be developed that measure disease-related perturbations 'downstream' of the initial causal factor. Fortunately, there are many animal models that can be used for this purpose. These include animals with specific defects in such pivotal genes as those encoding the transcription factor Foxp3 (refs. 11,12), interleukin 2, the kinase Zap70, Fas and other important tolerance mediators. The point is not that human T1D is due to defects in these specific genes but instead that each of the pathways identified by these models needs to be measured specifically in humans to stratify disease phenotypes appropriately.

Another category of mouse models of diabetes is those that are 'humanized', most often by the introduction of genes encoding MHC, T cell antigen receptors (TCRs) and costimulatory molecules from humans, sometimes with the intention of transferring lymphocytes for modeling disease initiation. The overarching goal of recreating human diabetes in a mouse is fraught with considerable difficulty, including recapitulation of *in vivo* properties of cell population expansion, homing and interactions with extracellular matrices and tissues that have not been humanized. Nevertheless, some intermediate goals are likely to be more approachable with these models, such as specifically evaluating *in vivo* immunomodulation by specific antigens presented in the context of humanized MHC and other genes[13,14].

In addition, promising alternative models now exist for addressing the issue of which T cells and antigens drive the diabetogenic response. As it has been established that a few islet antigen–specific T cells in a sea of nonspecific bystanders can drive complete beta-cell destruction[15], the question of whether there is a particular TCR that causes the demise of most beta cells or at least commonly triggers the process is very important. The technology now available does not yet allow easy and reliable detection of such cells in very low numbers in blood, and cause-effect relationships are obviously difficult to establish in humans. A rapid technique for inserting TCRs with monospecificity into the NOD diabetes model has provided some insight into potentially pro-diabetogenic (effector) or anti-diabetogenic clones of regulatory T cells[16]. However, such mice do not allow the observation and evaluation of a given TCR's function with more physiological T cell numbers, which must be taken into account when extrapolating these observations to the human situation. Confirmation of the pathogenic involvement of a single TCR will be important, perhaps by direct staining of islet sections of both mouse and man with peptide-MHC tetramers. In addition, systematic and rapid sequencing of the TCRs present in human insulitic lesions should be

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undertaken with advanced 'next-generation' DNA sequencing technology. Nevertheless, it is entirely possible that TCR usage is heterogeneous even in a given person, which would considerably diminish the feasibility of tracking autoaggressive T cells with one specificity or a few specificities as a biomarker for disease progression or therapeutic success.

The insertion of a target (marker) antigen into beta cells by transgenic technology has been widely used as an experimental approach for studying tolerance to beta-cell antigens since it was introduced in the late 1980s and early 1990s. The expression in beta cells of viral antigens from influenza and lymphocytic choriomeningitis virus[17] and other proteins unrelated to diabetes[18,19] (ovalbumin, and SV40 T antigen) has been pivotal in the development of an understanding of the relationship between thymic expression, peripheral presentation and cross-presentation, as well as the interaction of $CD4^+$ and $CD8^+$ T cells and B lymphocytes. Such models continue to be used for testing of various modalities of antigen specific tolerance. Because the initiating autoantigen(s) in human T1D is (are) still unknown, antigen-specific models such as these are important in delineating the pathways leading to and therapies for preventing beta-cell destruction. In particular, these models offer the opportunity to test interventions on diverse genetic backgrounds, which is probably important for gauging the potential success of a given intervention in human patients with T1D, who are genetically diverse.

Conclusions

Overall, the present landscape of basic and translational research in animal models of T1D is characterized by overuse of the NOD mouse. This scenario has some historic reasons that are understandable, foremost among those being the fact that NOD mice and humans share several susceptibility-related genes, including genes encoding the MHC class II homologs. But it is now also known that there is considerable complexity and heterogeneity in both the disease and in the genetics of the disease, and a singular focus on the NOD model generates too narrow a perspective. One area that further emphasizes this concern is the observations that human insulitis seems to be much milder than the islet infiltration present in most rodent models and that $CD8^+$ T cells can predominate in humans[20]. In addition, some studies have found upregulation of MHC class I and interferon-α in islets of T1D patients without accompanying T cell infiltrates, which might be a strong indicator (if independently confirmed) of viral involvement in the pathogenesis of human T1D[20]. Over the past two decades, the islet pathology of the NOD mouse, rather than human pathology, has driven and formed the understanding of T1D. This model has indisputably been extremely valuable in developing the present understanding of pathological effector and regulatory mechanisms, but the time is right for a broader reevaluation shaped by what is observed in humans, not by what is possible in mice.

The ultimate translational objective, of course, is to use these models for accelerating new therapeutics into implementation in humans for the prevention of and intervention in T1D. With this in mind, we have identified several areas of potential emphasis for future research focus.

Are there differences in the pathogenesis and response to therapy of mouse diabetes disease models with an acute onset versus those with a more progressive, relapsing-remitting disease onset? The latter are potentially valuable for the evaluation of clinical therapeutics in the context of human disease presentation, which can span 7–9 years of progressive autoimmunity before the development of clinical hyperglycemia[21].

What is the effect of combinations of 'predisposition genes' on particular immunological pathways that can be targeted with therapy? For example, the use of 'hit-and-run' viral causation models may be particularly relevant for triggering the spreading of determinants to islet antigens. Such studies might suggest lessons for B cell–directed therapeutics, whereas models of T cell–modulated responses might be particularly relevant to the stratification of patients on the basis of profiling of susceptibility-related genes for therapy with T cell–activation inhibitors.

Can the idea of heterogeneity be extended for better understanding of variation in the interaction of B cell, $CD4^+$ T cell and $CD8^+$ T cell responses and their pathogenic potential through a focus on specific objectives that can be addressed in humanized mice, including mice that have human islets, MHC molecules and TCRs? Little is understood about the function of macrophages, mast cells and various dendritic cell subsets during the pathogenesis of T1D, in the context of either disease augmentation or disease remission. However, no animal models have been shown to address these gaps.

Advances in cell 'programming' and differentiation are encouraging for progress in the area of beta-cell regeneration and repair, but these now must be paired with analysis in the context of antigen-specific autoimmunity. It is unclear whether islet replication increases during the pathogenesis of T1D, but it has been established in animal models that inflammatory factors can enhance beta-cell replication[22]. Precise quantification of the ratio of beta-cell apoptosis to beta-cell regeneration is lacking, and therefore the turnover of beta cells during the pathogenesis of human T1D is not known precisely. New models for tracking beta-cell neogenesis[23] are needed and should be coupled with immunological investigations.

Beta cells do not function in isolation, of course, and the potential for matrix-cellular interactions, neuronal interactions or other

Box 2

Challenges in human studies; opportunities for animal models

- Ease of access to pancreata and pancreatic lymph nodes for direct assessment of disease and/or response to therapy
- Determination of dose-dependent variables not predicted by *in vitro* studies, by evaluation of broad dose range and timing of experimental therapeutics
- Evaluation in the context of chronic infection, not just in normal healthy animals, to assess risk
- Direct comparison of alternative antigen-specific delivery modalities and therapies for peripheral tolerization
- Validation in multiple models to anticipate issues of human genetic heterogeneity

Next-generation animal models

- Human non-MHC T1D genes on H-2^{b7} 'diabetes-resistant' backgrounds to understand T1D genotype-phenotype relationships
- Knockout and overexpression models of specific immunomodulatory pathways for analysis of biomarkers and disease effect
- Humanized and retrogenic studies of human T cell specificity
- Focus of islet antigen-target transgenics on the development of insights for antigen-specific therapies, coupled with environmental and genotypic interactions to model human heterogeneity
- '*In silico*' virtual' mice

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homeostasis mechanisms to modulate islet health and response to injury should not be ignored. The immunology community has been slow to embrace models in which these types of manipulation of the pancreatic islets could be beneficial for the prevention, delay or treatment of T1D. In therapeutic preclinical studies, the effects of chronic immune modulation in the context of infection and even tumor-prone or nondiabetic autoimmune-prone hosts should be evaluated. Ultimately, safety concerns are likely to be the main determinant of therapeutic choice, and it would be better to conduct clinical trials armed with this knowledge.

We also acknowledge that not all obstacles to progress in this area are amenable to laboratory investigation. It is unknown how to properly equate the mouse lifespan to that of humans, so 1- or 2-year 'long-term' protection in a mouse could represent a fairly short 1- or 2-year interval for a teenage diabetic and thus may require repeated therapeutic administration. In addition, the academic climate and its present means of career evaluation and peer review are not optimized for efficiency in the clinical 'translation' of animal models. Experimental confirmation in multiple models is not considered cutting-edge research and is not favored in the peer-review system for publications or grants, yet it might be seminal for optimal assessment of the potential of a given therapy in an outbred human population. Larger team efforts and partial contributions to large teams are not rewarded by reviews requiring that young investigators demonstrate independence. The scientific cultural environment does not support comparative studies of therapeutic efficacy and safety and dose-finding investigations, and when such studies are done by 'industry', they are almost never published. However, such studies would not only improve the preclinical development pathway but also help focus the research community on relevant translational questions.

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EXHIBIT O

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Research in Translation

Can Animal Models of Disease Reliably Inform Human Studies?

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Animal experiments have contributed much to our understanding of mechanisms of disease, but their value in predicting the effectiveness of treatment strategies in clinical trials has remained controversial [1–3]. In fact, clinical trials are essential because animal studies do not predict with sufficient certainty what will happen in humans. In a review of animal studies published in seven leading scientific journals of high impact, about one-third of the studies translated at the level of human randomised trials, and one-tenth of the interventions, were subsequently approved for use in patients [1]. However, these were studies of high impact (median citation count, 889), and less frequently cited animal research probably has a lower likelihood of translation to the clinic. Depending on one's perspective, this attrition rate of 90% may be viewed as either a failure or as a success, but it serves to illustrate the magnitude of the difficulties in translation that beset even findings of high impact.

Recent examples of therapies that failed in large randomised clinical trials despite substantial reported benefit in a range of animal studies include enteral probiotics for the prevention of infectious complications of acute pancreatitis, NXY-059 for acute ischemic stroke, and a range of strategies to reduce lethal reperfusion injury in patients with acute myocardial infarction [4–7]. In animal models of acute ischemic stroke, about 500 "neuroprotective" treatment strategies have been reported to improve outcome, but only aspirin and very early intravenous thrombolysis with alteplase (recombinant tissue-plasminogen activator) have proved effective in patients, despite numerous clinical trials of other treatment strategies [8,9].

Linked Research Article

This Research in Translation discusses the following new study published in *PLoS Biology*:

Sena ES, van der Worp HB, Bath PMW, Howells DW, Macleod MR (2010) Publication bias in reports of animal stroke studies leads to major overstatement of efficacy. PLoS Biol 8(3): e1000344. doi:10.1371/journal.pbio.1000344

Publication bias confounds attempts to use systematic reviews to assess the efficacy of various interventions tested in experiments modeling acute ischemic stroke, leading to a 30% overstatement of efficacy of interventions tested in animals.

Causes of Failed Translation

The disparity between the results of animal models and clinical trials may in part be explained by shortcomings of the clinical trials. For instance, these may have had insufficient statistical power to detect a true benefit of the treatment under study. For practical or commercial purposes, the designs of some clinical trials have also failed to acknowledge the limitations of efficacy observed in animal studies, for example by allowing therapy at later time points when the window of opportunity has passed [10,11]. Secondly, the failure of apparently promising interventions to translate to the clinic may also be caused by inadequate animal data and overoptimistic conclusions about efficacy drawn from methodologically flawed animal studies. A third possible explanation is the lack of external validity, or generalisability, of some animal models; in other words, that these do not sufficiently reflect disease in humans. Finally, neutral or negative animal studies may be more likely to remain unpublished than neutral clinical trials, giving the impression that the first are more often positive than the second. This article aims to address the possible sources of bias that threaten the internal and external validity of animal studies, to provide solutions to improve the reliability of such studies, and thereby to improve their translation to the clinic.

Internal Validity

Adequate internal validity of an animal experiment implies that the differences observed between groups of animals

Research in Translation discusses health interventions in the context of translation from basic to clinical research, or from clinical evidence to practice.

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Abbreviations: ALS, amyotrophic lateral sclerosis; CAMARADES, Collaborative Approach to Meta-Analysis And Review of Animal Data from Experimental Stroke; CONSORT, CONsolidated Standards Of Reporting Trials

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Summary Points

- The value of animal experiments for predicting the effectiveness of treatment strategies in clinical trials has remained controversial, mainly because of a recurrent failure of interventions apparently promising in animal models to translate to the clinic.
- Translational failure may be explained in part by methodological flaws in animal studies, leading to systematic bias and thereby to inadequate data and incorrect conclusions about efficacy.
- Failures also result because of critical disparities, usually disease specific, between the animal models and the clinical trials testing the treatment strategy.
- Systematic review and meta-analysis of animal studies may aid in the selection of the most promising treatment strategies for clinical trials.
- Publication bias may account for one-third or more of the efficacy reported in systematic reviews of animal stroke studies, and probably also plays a substantial role in the experimental literature for other diseases.
- We provide recommendations for the reporting of aspects of study quality in publications of comparisons of treatment strategies in animal models of disease.

allocated to different interventions may, apart from random error, be attributed to the treatment under investigation [12]. The internal validity may be reduced by four types of bias through which systematic differences between treatment groups are introduced (Table 1). Just like any clinical trial, each formal animal study testing the effectiveness of an intervention should be based on a well-designed study protocol addressing the design and conduct of the study, as well as the analysis and reporting of its results. Aspects of the design, conduct, and analysis of an animal experiment that help to reduce bias and to improve the reliability and reproducibility of the results are discussed below. As the impact of study quality has been studied much more extensively in clinical trials than in animal studies, the backgrounds and recommendations regarding these issues are largely based on the clinical CONsolidated Standards of Reporting Trials (CONSORT) statement, and to a smaller extent on published recommendations and guidelines for the conduct and reporting of animal studies of acute ischemic stroke [13–17].

Randomisation

To prevent selection bias, treatment allocation should be based on randomisation (Box 1), a method that is almost ubiquitous in clinical treatment trials. In part, this prevents the investigator from having to choose which treatment a particular animal will receive, a process which might result (consciously or subconsciously) in animals which are thought to do particularly well or particularly badly being overrepresented in a particular treatment group. Foreknowledge of treatment group assignment may also lead to selective exclusion of animals based on prognostic factors [13]. These problems can arise with any method in which group allocation is known in advance or can be predicted. Such methods include both the use of predetermined rules (e.g., assignment in alternation or on the basis of the days of the week) or of open randomisation schedules. Picking animals "at random" from their cages also has the risk of conscious or subconscious manipulation, and does not represent true randomisation.

Randomisation may appear redundant if the animals form a homogeneous group from a genetic and environmental perspective, as often is the case with rats and other rodents. However, it is not only the animal itself but mainly the induction of the disease that may give rise to variation. For example, there is a large variation in infarct size in most rat models of ischaemic stroke not only because of interindividual differences in collateral circulation—even in inbred strains—but also because in some animals the artery is occluded better than in others and because the models are inherently vulnerable to complications that may affect outcome, such as periprocedural hypotension or hypoxemia. It is because of this variation that randomisation, ideally occurring after the injury or disease has been induced, is essential.

In clinical trials, automated randomisation techniques such as random number generation are most commonly used, but manual methods (such as tossing a coin or throwing dice) are also acceptable as long as these cannot be manipulated. By preference, such manual techniques should be performed by an independent person.

Blinding

In studies that are blinded throughout their course, the investigators and other persons involved will not be influenced by knowledge of the treatment assignment, thereby preventing performance, detection, and attrition bias. Knowledge of treatment assignment may subconsciously or otherwise affect the supply of additional care, outcome assessment, and decisions to withdraw animals from the experiment.

In contrast to allocation concealment (Box 1), blinding may not always be possible in all stages of an experiment, for example when the treatment under investigation concerns a surgical proce-

Table 1. Four types of bias threatening internal validity.

Type of Bias	Definition	Solution
Selection bias	Biased allocation to treatment groups	Randomisation; allocation concealment
Performance bias	Systematic differences in care between the treatment groups, apart from the intervention under study	Blinding
Detection (ascertainment, assessment, or observer) bias	Systematic distortion of the results of a study that occurs when the person assessing outcome has knowledge of treatment assignment	Blinding
Attrition bias	Unequal occurrence and handling of deviations from protocol and loss to follow-up between treatment groups	Blinding; intention-to-treat analysis

Adapted from [12,13].

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Box 1. Glossary

- **Allocation concealment:** Concealing the allocation sequence from those assigning animals to intervention groups, until the moment of assignment.
- **Bias:** Systematic distortion of the estimated intervention effect away from the "truth," caused by inadequacies in the design, conduct, or analysis of an experiment.
- **Blinding (masking):** Keeping the persons who perform the experiment, collect data, and assess outcome unaware of the treatment allocation.
- **Eligibility criteria:** Inclusion and exclusion criteria: the characteristics that define which animals are eligible to be enrolled in a study.
- **External validity:** The extent to which the results of an animal experiment provide a correct basis for generalisations to the human condition.
- **Intention-to-treat analysis:** Analysis of data of all animals included in the group to which they were assigned, regardless of whether they completed the intervention.
- **Internal validity:** The extent to which the design and conduct of the trial eliminate the possibility of bias.
- **Power:** The probability that a study will detect a statistically significant effect of a specified size.
- **Randomisation:** Randomly allocating the intervention under study across the comparison groups, to ensure that group assignment cannot be predicted.
- **Sample size:** The number of animals in the study.

Definitions adapted from [13] and from Wikipedia (http://www.wikipedia.org, accessed on 9 November 2009).

dure. However, blinding of outcome assessment is almost always possible.

In clinical trials, the most common form of blinding is double blinding, in which the patients, the investigators, and the caregivers are unaware of the intervention assignment. Because the patient does not know which treatment is being administered, the placebo effect will be similar across the comparison groups. As animals are not susceptible to the placebo effect, double blinding is not an issue in animal studies. Notwithstanding the influence that unblinded animal handling can have on performance in neurobehavioural tasks [18], the fact that in some articles of animal studies "double blinding" is reported raises questions about the authors' knowledge of blinding as well as about the review and editorial processes of the journals in which the studies were published [19,20].

Sample Size Calculation

Selection of target sample size is a critical factor in the design of any comparison study. The study should be large enough to have a high probability of detecting a treatment effect of a given size if such an effect truly exists, but also pay attention to legal requirements and ethical and practical considerations to keep the number of animals as small as possible. The required sample size should be determined before the start of the study with a formal sample size calculation, of which the fundamental elements of statistical significance (α), effect size (δ), power ($1-\beta$), and standard deviation of the measurements have been explained in numerous articles [13,21]. Unfortunately, the assumptions on variation of the measurements are often based on incomplete data, and small errors can lead to a study that is either under- or overpowered. From an ethical point of view, underpowered studies are undesirable, as they might lead to the false conclusion that the intervention is without efficacy, and all included animals will have been used to no benefit. Overpowered studies would also be unethical, but these are much less prevalent.

Monitoring of Physiological Parameters

Depending on the disease under investigation, a range of physiological variables may affect outcome, and inadequate control of these factors may lead to erroneous conclusions. Whether or not physiological parameters should be assessed, and for how long, therefore depends on the model and on the tested condition.

Eligibility Criteria and Drop-Outs

Because of their complexity, many animal models are inherently vulnerable to complications—such as inadvertent blood loss during surgery to induce cerebral or myocardial ischemia—that are not related to the treatment under study but that may have a large effect on outcome. Given the explanatory character of preclinical studies, it is justifiable to exclude animals with such complications from the analyses of treatment effects, provided that the eligibility criteria are predefined and not determined on a post-hoc basis, and that the person responsible for the exclusion of animals is unaware of the treatment assignment.

In clinical trials, inclusion and exclusion criteria are usually applied before enrolment in the study, but for the reason above, in animal studies it is justifiable also to apply these criteria during the course of the study. However, these should be limited to complications that are demonstrably not related to the intervention under study, as this may otherwise lead to attrition bias. For example, if a potential novel treatment for colorectal cancer increases instead of reduces tumour progression, thereby weakening the animals and increasing their susceptibility to infections, exclusion of animals dying prematurely because of respiratory tract infections may lead to selective exclusion of animals with the largest tumours and mask the detrimental effect of the novel intervention.

Statistical Analysis

The statistical analysis of the results of animal experiments has been given elaborate attention in review articles and books [22]. However, even when data appear simple and their analysis straightforward, inadequate techniques are often used. Common examples include the use of a *t*-test for nonparametric data, calculating means and standard deviations for ordinal data, and treating multiple observations from one animal as independent.

In clinical trials, an intention-to-treat analysis is generally favoured because it avoids bias associated with nonrandom loss of participants [13]. As explained above, the explanatory character of most studies justifies the use of an analysis restricted to data from animals that have fulfilled all eligibility criteria, provided that all animals excluded from the analysis are accounted for and that those exclusions have been made without knowledge of treatment group allocation.

Control of Study Conduct

The careers of investigators at academic institutions and in industry depend in part on the number and impact of their publications, and these investigators may be all too aware of the fact that the prospect of their work being published

increases when positive results are obtained. This underscores not only the importance of randomisation, allocation concealment, and blinding, but also the need for adequate monitoring and auditing of laboratory experiments by third parties. Indeed, adopting a multicentre approach to animal studies has been proposed, as a way of securing transparent quality control [23].

Bias in Animal Studies

The presence of bias in animal studies has been tested most extensively in studies of acute ischemic stroke, probably because in this field the gap between the laboratory and the clinic is both very large and well recognised [8]. In systematic reviews of different interventions tested in animal models of acute ischemic stroke, other emergencies, Parkinson's disease, multiple sclerosis, or amyotrophic lateral sclerosis, generally about a third or less of the studies reported random allocation to the treatment group, and even fewer studies reported concealment of treatment allocation or blinded outcome assessment [2,16,19,24,25]. Even when reported, the methods used for randomisation and blinding were rarely given. A priori sample size calculations were reported in 0%–3% of the studies (Table 2).

Complications of the disease and/or treatment under study were reported in 19% of the studies of hypothermia for acute ischemic stroke. All but one of these complications concerned premature death, and about 90% of these animals were excluded from the analyses [20]. In another review of several treatment strategies for acute ischemic stroke, only one of 45 studies mentioned predefined inclusion and exclusion criteria, and in just 12 articles (27%) exclusion of animals from analysis was mentioned and substantiated. It is difficult to believe that in every other study every single experiment went as smoothly as the investigators had planned [19].

Two factors limit the interpretation of the above-mentioned data. First, the assessment of possible confounders in systematic reviews was based on what was reported in the articles, and may have been incomplete because the authors considered these aspects of study design not sufficiently relevant to be mentioned. In addition, definitions of randomisation, allocation concealment, and blinding might vary across studies, and, for example, randomly picking animals from their cages may have been called "randomisation." Indeed, a survey of a sample of authors of publications included in such reviews suggested that this was sometimes the case [26].

Quality Checklists

At least four different but largely overlapping study-quality checklists have been proposed for use in animal studies of focal cerebral ischemia. These checklists have included items relating first to the range of circumstances under which efficacy has been shown and second to the characteristics that might act as a source of bias in individual experiments [16].

Assessment of overall methodological quality of individual studies with these checklists is limited by controversy about the composition of the checklists and, more importantly, because the weight of each of the individual components has remained uncertain. For example, in the most frequently used CAMARADES checklist, "adequate allocation concealment" may have a much larger impact on effect size than "compliance with regulatory requirements" [16].

Does Methodological Quality Matter?

Several systematic reviews and meta-analyses have provided empirical evidence that inadequate methodological approaches in controlled clinical trials are associated with bias. Clinical trials in which authors did not report randomisation, adequately conceal treatment allocation, or use double blinding yielded larger estimates of treatment effects than trials in which these study quality issues were reported [12,27–32].

Table 2. Randomisation, blinded outcome assessment, and sample size calculation in systematic reviews of animal studies.

Disease Modeled	Year of Publication	Number of Publications	Randomisation, *n* (%)	Blinded Outcome Assessment, *n* (%)	A Priori Sample Size Calculation, *n* (%)
Heart failure [24]	2003	9	6 (67)	9 (100)	0 (0)
Emergency medicine [33]	2003	290	94 (32)	31 (11)	N/A
Ischemic stroke [19]	2005	45	19 (42)	18 (40)	0 (0)
Ischemic stroke [49]	2005	73	17 (23)	9 (12)	N/A
Ischemic stroke [50]	2005	25	8 (32)	1 (4)	N/A
Ischemic stroke [51]	2006	27	2 (7)	1 (4)	N/A
Traumatic brain injury [2]	2007	17	2 (12)	3 (18)	N/A
Hemorrhage in surgery [2]	2007	8	3 (38)	4 (50)	N/A
Neonatal RDS [2]	2007	56	14 (25)	3 (5)	N/A
Osteoporosis [2]	2007	16	5 (31)	0 (0)	N/A
Ischemic stroke [16]*	2007	288	103 (36)	84 (29)	8 (3)
Parkinson's disease [16]	2007	118	14 (12)	18 (15)	0 (0)
Multiple sclerosis [16]	2007	183	4 (2)	20 (11)	0 (0)
ALS [45]	2007	85	21 (25)	21 (25)	1 (1)
Brain injury [52]	2008	18	12 (67)	7 (39)	N/A
Ischemic stroke [25]	2008	9	3 (33)	4 (44)	2 (22)
Ischemic stroke [53]	2009	19	1 (5)	5 (26)	0 (0)

*Summarises the data of six systematic reviews of treatment strategies for acute ischemic stroke. There is an overlap of 18 publications between references [16] and [19].
ALS, amyotrophic lateral sclerosis; N/A, data not available; RDS, respiratory distress syndrome.
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The impact of methodological quality on the effect size in animal studies has been examined less extensively. In animal studies testing interventions in emergency medicine, the odds of a positive result were more than three times as large if the publication did not report randomisation or blinding as compared with publications that did report these methods [33]. In systematic reviews of FK-506 or hypothermia for acute ischemic stroke, an inverse relation was found between effect size and study quality, as assessed by a ten-item study-quality checklist [20,34]. The same review on hypothermia found large overstatements of the reduction in infarct volume in animal stroke studies without randomisation or blinded outcome assessment when they were compared with randomised or blinded studies, but a meta-analysis of 13 meta-analyses in experimental stroke describing outcomes in a total of 15,635 animals found no statistically significant effect of these quality items on effect size. In this meta-meta-analysis, only allocation concealment was associated with a larger effect size [35].

A limitation of the meta-analyses assessing the effect of study quality aspects on effect size is the fact that no consideration has been given to possible interactions between quality items, and that only univariate analyses were performed. However, individual quality aspects that may affect the results of meta-analyses of animal studies are unlikely to operate independently. For example, nonrandomised studies may be more likely than randomised studies to disregard other quality issues, such as allocation concealment or blinding, or to use shorter delays for the initiation of treatment, all of which may affect study results. The relative importance of the various possible sources of bias is therefore not yet known and is the subject of ongoing research.

External Validity

Even if the design and conduct of an animal study are sound and eliminate the possibility of bias, the translation of its results to the clinic may fail because of disparities between the model and the clinical trials testing the treatment strategy. Common causes of such reduced external validity are listed in Box 2 and are not limited to differences between animals and humans in the pathophysiology of disease, but also include differences in comorbidities, the use of co-medication, timing of the administration and dosing of the study treatment, and the selection of outcome measures. Whereas the issues for internal validity probably apply to the majority of animal models regardless of the disease under study, the external validity of a model will largely be determined by disease-specific factors.

Box 2. Common Causes of Reduced External Validity of Animal Studies

- The induction of the disease under study in animals that are young and otherwise healthy, whereas in patients the disease mainly occurs in elderly people with co-morbidities.
- Assessment of the effect of a treatment in a homogeneous group of animals versus a heterogeneous group of patients.
- The use of either male or female animals only, whereas the disease occurs in male and female patients alike.
- The use of models for inducing a disease or injury with insufficient similarity to the human condition.
- Delays to start of treatment that are unrealistic in the clinic; the use of doses that are toxic or not tolerated by patients.
- Differences in outcome measures and the timing of outcome assessment between animal studies and clinical trials.

Stroke Models

As mentioned above, the translation of efficacy from animal studies to human disease has perhaps been least successful for neurological diseases in general and for ischaemic stroke in particular. As there is also no other animal model of disease that has been more rigorously subjected to systematic review and meta-analysis, stroke serves as a good example of where difficulties in translation might arise.

The incidence of stroke increases with age, and stroke patients commonly have other health problems that might increase their stroke risk, complicate their clinical course, and affect functional outcome. Of patients with acute stroke, up to 75% and 68% have hypertension and hyperglycaemia, respectively [9,36]. While it is important to know whether candidate stroke drugs retain efficacy in the face of these comorbidities, only about 10% of focal ischaemia studies have used animals with hypertension, and fewer than 1% have used animals with induced diabetes. In addition, animals used in stroke models were almost invariably young, and female animals were highly underrepresented. Over 95% of the studies were performed in rats and mice, and animals that are perhaps biologically closer to humans are hardly ever used [16,19]. Moreover, most animal studies have failed to acknowledge the inevitable delay between the onset of symptoms and the possibility to start treatment in patients. In a systematic review of animal studies of five different neuroprotective agents that had also been tested in 21 clinical trials including a total of more than 12,000 patients with acute ischaemic stroke, the median time between the onset of ischaemia and start of treatment in the animal studies was just 10 minutes, which is infeasible in the clinic [19]. In the large majority of clinical trials, functional outcome is the primary measure of efficacy, whereas animal studies usually rely on infarct volume. Several studies have suggested that in patients the relation between infarct volume and functional outcome is moderate at best [37,38]. Finally, the usual time of outcome assessment of 1–3 days in animal models contrasts sharply with that of 3 months in patients [19]. For these reasons, it is not surprising that, except for thrombolysis, all treatment strategies proven effective in the laboratory have failed in the clinic.

Other Acute Disease Models

Differences between animal models and clinical trials similar to those mentioned above have been proposed as causes of the recurrent failure of a range of strategies to reduce lethal reperfusion injury in patients with acute myocardial infarction [6,7]. The failure to acknowledge the presence of often severe comorbidities in patients, and short and clinically unattainable onset-to-treatment delays, have also limited the external validity of animal models of traumatic brain injury [2].

Chronic Disease Models

The external validity of models of chronic and progressive diseases may also be challenged by other factors. For the treatment of Parkinson's disease, researchers have mainly relied on injury-induced models that mimic nigrostriatal dopamine deficiency but do not recapitulate the slow, progressive, and degenerative nature of the disease in humans. Whereas in clinical trials interventions were administered over

a prolonged period of time in the context of this slowly progressive disease, putative neuroprotective agents were administered before or at the same time as an acute Parkinson's disease-like lesion was induced in the typical underlying animal studies [39].

Based on the identification of single point-mutations in the gene encoding superoxide dismutase 1 (SOD1) in about 3% of the patients with amyotrophic lateral sclerosis (ALS), mice carrying 23 copies of the human SOD1G93A transgene are considered the standard model for therapeutic studies of ALS. Apart from the fact that this model may be valid only for patients with SOD1 mutations, the mice may suffer from a phenotype that is so aggressive and so overdriven by its 23 copies of the transgene that no pharmacological intervention outside of the direct inhibition of SOD1 will ever affect ALS-related survival. In addition, it has been suggested that these mice may be more susceptible to infections and other non-ALS related illnesses and that it is this illness rather than the ALS that is alleviated by the experimental treatment. Consistent with this hypothesis, several of the compounds reported as efficacious in SOD1G93A mice are broad-spectrum antibiotics and general anti-inflammatory agents [40].

Publication Bias

Decisions to assess the effect of novel treatment strategies in clinical trials are, ideally, based on an understanding of all publicly reported information from preclinical studies. Systematic review and meta-analysis are techniques developed for the analysis of data from clinical trials and may be helpful in the selection of the most promising strategies [16]. However, if studies are published selectively on the basis of their results, even a meta-analysis based on a rigorous systematic review will be misleading.

The presence of bias in the reporting of clinical trials has been studied extensively. There is strong empirical evidence that clinical studies reporting positive or significant results are more likely to be published, and that outcomes that are statistically significant have higher odds of being reported in full rather than as an abstract. Such publication bias will lead to overestimation of treatment effects and can make the readily available evidence unreliable for decision making [41].

Unfortunately, the presence of publication bias in animal studies has received much less attention. In a recent systematic review of studies testing the efficacy of interventions in animal models of human disease, only six reported testing for the presence of publication bias, and such bias was found in four [34,42–46]. No study gave quantitative estimates of the impact on effect size of publication bias [47].

In a subsequent meta-analysis of 525 publications [47] included in systematic reviews of 16 interventions tested in animal studies of acute ischaemic stroke, Egger regression and Trim and Fill analysis suggested that publication bias was widely prevalent. The analyses suggested that publication bias might account for around one-third of the efficacy reported in systematic reviews of animal stroke studies. Because this meta-analysis included all reported experiments testing an effect of an intervention on infarct size, and not just the experiment with the largest effect size from each publication, at least some experiments testing ineffective doses (e.g., at the lower end of a dose-response curve) were included. For this reason, this meta-analysis is more likely to underestimate than to overestimate the effect of publication bias. It is therefore probably more revealing that of the 525 publications, only ten (2%) did not report at least one significant effect on either infarct volume or neurobehavioural score [47]. Although unproven, it appears unlikely that the animal stroke literature is uniquely susceptible to publication bias.

Nonpublication of the results of animal studies is unethical not only because it deprives researchers of the accurate data they need to estimate the potential of novel therapies in clinical trials, but also because the included animals are wasted because they do not contribute to accumulating knowledge. In addition, research syntheses that overstate biological effects may lead to further unnecessary animal experiments testing poorly founded hypotheses.

Practical Improvement Strategies

Although there is no direct evidence of a causal relationship, it is likely that the recurrent failure of apparently promising interventions to improve outcome in clinical trials has in part been caused by inadequate internal and external validity of preclinical studies and publication bias favouring positive studies. On the basis of ample empirical evidence from clinical trials and some evidence from preclinical studies, we suggest that the testing of treatment strategies in animal models of disease and its reporting should adopt standards similar to those in the clinic to ensure that decision making is based on high-quality and unbiased data. Aspects of study quality that should be reported in any manuscript are listed in Box 3.

Not only should the disease or injury itself reflect the condition in humans as much as possible, but age, sex, and comorbidities should also be modelled where possible. The investigators should

Box 3. Aspects of Study Quality to Be Reported in the Manuscript

- **Sample size calculation:** How the sample size was determined, and which assumptions were made.
- **Eligibility criteria:** Inclusion and exclusion criteria for enrolment.
- **Treatment allocation:** The method by which animals were allocated to experimental groups. If this allocation was by randomisation, the method of randomisation.
- **Allocation concealment:** The method to implement the allocation sequence, and if this sequence was concealed until assignment.
- **Blinding:** Whether the investigators and other persons involved were blinded to the treatment allocation, and at which points in time during the study.
- **Flow of animals:** Flow of animals through each stage of the study, with a specific attention to animals excluded from the analyses. Reasons for exclusion from the analyses.
- **Control of physiological variables:** Whether and which physiological parameters were monitored and controlled.
- **Control of study conduct:** Whether a third party controlled which parts of the conduct of the study.
- **Statistical methods:** Which statistical methods were used for which analysis.

Recommendations based on [13,17].

Five Key Papers in the Field

Hackam 2006 [1]: Shows that about a third of highly cited animal research translates at the level of human randomised trials.

Sena 2007 [16]: Proposes minimum standards for the range and quality of pre-clinical animal data before these are taken to clinical trials.

Dirksen 2007 [6]: Provides an overview of the various strategies that inhibit reperfusion injury after myocardial infarction and discusses potential mechanisms that may have contributed to the discrepancy between promising pre-clinical data and the disappointing results in randomised clinical trials.

Scott 2008 [40]: Elaborate study suggesting that the majority of published effects of treatments for amyotrophic lateral sclerosis are most likely measurements of noise in the distribution of survival means as opposed to actual drug effect.

Sena 2010 [47]: The first study to estimate the impact of publication bias on the efficacy reported in systematic reviews of animal studies.

justify their selection of the model and outcome measures. In turn, human clinical trials should be designed to replicate, as far as is possible, the circumstances under which efficacy has been observed in animals. For an adequate interpretation of the potential and limitations of a novel treatment strategy, a systematic review and meta-analysis of all available evidence from preclinical studies should be performed before clinical trials are started. Evidence of benefit from a single laboratory or obtained in a single model or species is probably not sufficient.

Finally, the recognition of substantial publication bias in the clinical literature has led to the introduction of clinical trial registration systems to ensure that those summarising research findings are at least aware of all relevant clinical trials that have been performed [48]. Given that a framework regulating animal experimentation already exists in many countries, we suggest that this might be exploited to allow the maintenance of a central register of experiments performed, and registration referenced in publications.

Author Contributions

ICMJE criteria for authorship read and met: HBvdW DWH ESS MJP SR VO MRM. Wrote the first draft of the paper: HBvdW. Contributed to the writing of the paper: DWH ESS SR VO MRM.

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3. Hackam DG (2007) Translating animal research into clinical benefit. B M J 334: 163–164.
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7. Yellon DM, Hausenloy DJ (2007) Myocardial reperfusion injury. N Engl J Med 357: 1121–1135.
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15. Dirnagl U (2006) Bench to bedside: the quest for quality in experimental stroke research. J Cereb Blood Flow Metab 26: 1465–1478.
16. Sena E, Van der Worp HB, Howells D, Macleod M (2007) How can we improve the pre-clinical development of drugs for stroke? Trends Neurosci 30: 433–439.
17. Macleod MR, Fisher M, O'Collins V, Sena ES, Dirnagl U, et al. (2009) Good laboratory practice: preventing introduction of bias at the bench. Stroke 40: e50–e52.
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20. Van der Worp HB, Sena ES, Donnan GA, Howells DW, Macleod MR (2007) Hypothermia in animal models of acute ischaemic stroke: a systematic review and meta-analysis. Brain 130: 3063–3074.
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surrogate or auxiliary outcome measure in ischemic stroke clinical trials. Stroke 30: 288–298.
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EXHIBIT P

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Habib Zaghouani, et al.	Title:	Sustained Treatment of Type 1 Diabetes After Expression of Predisposition Markers
App. No.:	10/681,788	Art Unit:	1644
Conf. No.:	6701	Examiner:	Edwoldt, Gerald R.
Filing Date:	October 8, 2003		

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

I, Habib Zaghouani, do hereby declare and say:

1. I am a citizen of the United States and my current residential address is 1608 Brookfield Manor, Columbia, Missouri, 65203.
2. I obtained my undergraduate degree in biochemistry from University of Paris, France in 1981. I obtained a Ph.D. in immunology from the University of Paris/Cancer Research Institute, France in 1987.
3. I am presently the J. Lavenia Edwards Chair in Pediatrics, Director, Center for Cellular and Molecular Immunology and Professor, Department of Molecular Microbiology & Immunology and Department of Child Health at the University of Missouri.
4. I have over one hundred publications and abstracts in the field of immunology. Please refer to the copy of my *curriculum vitae* in attached Appendix A for more details.
5. I am a named inventor on the '788 application as well as on related co-pending application serial numbers: 10/510,411; 11/290,070; and 11/425,084.
6. I have performed an experiment examining the impact of administration, initiated at the pre-diabetic stage, of soluble Ig-GAD2 to NOD mice over a period of 56 weeks. Data are provided in attached Appendix B.
7. NOD mice were assessed for blood glucose beginning at week 12 of age. Those mice that reached glucose levels of 160 – 250 mg/dl between week 14 to 25 received the following Ig-GAD2 regimen: 500 μg of soluble Ig-GAD2 i.p. daily for 5 days and then weekly injections thereafter for either 15 or 25 weeks. Blood glucose monitoring was performed during this period.
8. Overall, 100% of mice that became pre-diabetic at the age of 14 – 25 weeks and that were not treated with Ig-GAD2 progressed to diabetes (blood sugar level 300 mg/dl glucose) within 5 weeks after diagnosis of the pre-diabetic stage. Moreover, 60% of mice undergoing the 15-week treatment regimen were protected against diabetes throughout the

25 week post-hyperglycemia monitoring period. Interestingly, one mouse (Figure 1 B, left panel, open stars) progressed to diabetes by 5 weeks of treatment and 3 mice (Figure 1 B, plus, open diamond, and open pentagon) had similar disease manifestations shortly after interruption of the treatment.

9. When the regimen was extended to 25 weeks, 100% of the Ig-GAD2 treated animals were protected (Figure 1 A, right panel) and normoglycemia was restored in all mice (Figure 1 B, right panel). This status persisted throughout the duration of the study, which was terminated when the mice were 52 to 56 weeks of age.

10. Blood glucose levels for sol Ig-GAD2 (through week 24 of treatment) treated mice are shown in Table 1. Blood glucose levels for the untreated mice are shown in Table 2.

11. It is my professional opinion that the NOD mouse model is an appropriate and well accepted animal model for Type I diabetes.

12. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

December 19, 2007

Date

Habib Zaghouani, PhD

APPENDIX A

Curriculum Vitae Habib Zaghouani

EDUCATION

Ph.D.	1987	Immunology, University of Paris/Cancer Research Institute, Paris, France.
M.S.	1983	Immunology, University of Paris/Pasteur Institute, Paris, France.
B.S.	1981	Biochemistry, University of Paris, Paris, France.

POSITIONS AND RESEARCH EXPERIENCE

2006-present Director, Center for Cellular and Molecular Immunology, The University of Missouri School of Medicine, Columbia, MO

2006-present: J. Lavenia Edwards Chair in Pediatrics, the University of Missouri School of Medicine, Columbia, MO

2006-present: Professor, Department of Child Health, the University of Missouri School of Medicine, Columbia, MO.

2001-present: Professor, Department of Molecular Microbiology and Immunology, the University of Missouri School of Medicine, Columbia, MO.

2000-2001: Associate Professor, Department of Microbiology, the University of Tennessee, Knoxville, Tennessee.

1994-2000: Assistant Professor, Department of Microbiology, the University of Tennessee, Knoxville, Tennessee.

1990-1994: Research Assistant Professor, Department of Microbiology, Mount Sinai School of Medicine, New York.

1987-1989: Postdoctoral Fellow, Department of Microbiology, Mount Sinai School of Medicine, New York. Mentor: Dr. Constantin A. Bona.

1983-1987: Graduate Research Assistant, Ph.D. candidate, Immunology, University of Paris/Cancer Research Institute, Paris, France. Mentor: Dr. Marc Stanislawski.

1981-1983: Graduate Research Assistant, M.S. candidate, Immunology, Pasteur Institute, Paris, France. Director: Dr. Arthur Dony Strosberg.

RESEARCH GRANT SUPPORT

A. Principal Investigator

Active

1). 2RO1 NS 037406, National Institutes of Health, March 2004 - February 2009. Modulation of autoreactive T cells. PI: Habib Zaghouani.

2). 1RO1 DK 065748, National Institutes of Health, April 2005-March 2008. Immune tolerance against type I diabetes in mice. PI: Habib Zaghouani.

3). 2RO1 AI 48541, National Institutes of Health, May 2006- April 2011. Regulation of neonatal immunity. PI: Habib Zaghouani.

4). 1R21 AI 068746, National Institutes of Health. July 2007 – June 2009. Mimotopes against type I diabetes. PI: Habib Zaghouani.

Pending

1). 1RO1 NS057194-A2, National Institutes of Health, April 2008 - March 2013. Regulation of autoimmune encephalomylitis. PI: Habib Zaghouani.

2). 2RO1 DK 065748-01, National Institutes of Health, April 2008-March 2013. Immune tolerance against type I diabetes in mice. PI: Habib Zaghouani.

B. Co-investigator, Mentor, or Key Personnel

Active

T32 GM008396, National Institute of General Medical Sciences (NIGMS), July 1991-June 2012. Molecular Basis of Gene Expression and Signal Processing. PI: Mark Hannink (Zaghouani: Mentor).

T32 RR007004, National Institutes of Health, July 2005-June 2010, Postdoctoral Training in Comparative Medicine. PI: Craig Franklin (Zaghouani: Mentor).

T90 DK71510, National Institutes of Health, September 2004 – August 2009. Bench and Back: Clinical biodetectives training. PI: Mark Milanick (Zaghouani: Mentor).

R90 DK71510, National Institutes of Health, September 2004 – August 2009. Bench and Back: Clinical biodetectives training. PI: Mark Milanick (Zaghouani: Mentor).

KO8 AR048671, National Institutes of Health, June 2005-April 2008, Cytokine regulation of collagen-induced arthritis. PI: Robert Ortman (Zaghouani: Mentor).

1G20 RR021327, National Institutes of Health, September 2004-August 2009. Equipment for the MU Life Sciences Center. PI: Lon Dixon, (Zaghouani: Key personnel).

1 G20 RR019711, National Institutes of Health, September 2004-Agust 2009. Renovation of MU Medical School Vivarium. PI: Lon Dixon. (Zaghouani: Key personnel).

U19AT003264-01, National Institutes of Health, September 2005 – August 2009. TICIPS: HIV/AIDS, Secondary Infections and Immune Modulation. Center grant. PI: William Folk (Zaghouani: Faculty Member).

Research Foundation Grant, Arthritis Foundation, April 2006 – May 2008. Synoviolin is a target for arthritis. PI: Deyu Fang (Zaghouani: Mentor).

C. Previous Support (PI: Zaghouani, H)

1). R21 AI 062796, National Institutes of Health, July 2005-June 2007. Immune tolerance in the newborn mouse. Yearly direct cost $150,000. PI: Habib Zaghouani. No cost extension 11/30/2007

2). 1RO1 AI48541, National Institutes of Health, May 2001- April 2006. Regulation of neonatal immunity. Yearly direct cost: $175,000. PI: Habib Zaghouani.

3). Astral Inc, October 2001- September 2004. Development of Approaches to Combat Autoimmunity. PI: Habib Zaghouani.

4). RO1NS37406, National Institutes of Health, January 2000- December 2004. Modulation of autoreactive T cells. PI: Habib Zaghouani

5). RG2967B-3, National Multiple Sclerosis Society, October 2002 – March 31, 2004 Down-regulation of encephalitogenic T cells. PI: Habib Zaghouani.

6). RG2967A2/1, National Multiple Sclerosis Society, April 99 - March 2002. Down-regulation of encephalitogenic T cells. PI: Habib Zaghouani.

7). Astral Inc: March 95 - July 2001. A novel approach to delete encephalitogenic T cells. PI: Habib Zaghouani.

8). RG2778A1/1, National Multiple Sclerosis, April 96 - March 1999. A deletional strategy for encephalitogenic T cells. PI: Habib Zaghouani.

9). Astral Inc:, September 97- August 99. Generation of human Ig chimeras carrying wild type or antagonist forms of myelin peptides. PI: Habib Zaghouani.

10). 1R41AI47496, (STTR): National Institutes of Health, September 2000-August 2001. Treatment of EAE using a novel delivery system. . Co-PI: Habib Zaghouani.

TEACHING EXPERIENCE

2004:	Microbiology 205 (Medical Microbiology) 3 credit hours, 8 lecture contact hours, 170 student, Spring semester, University of Missouri School of Medicine, Columbia.
2002-present:	Microbiology 304 (Immunology) 3 credit hours, 14 lecture contact hours, 30 students, Fall semester, Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia.
2002-present	Microbiology 407 (advanced Immunology) 4 credit hours, 9 lecture contact hours, 18 students, Spring semester, Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia.
2001-present:	Bio 4952, Undergraduate research, 3 credit hours, 1-2 students, Fall and Winter semesters
2001-present:	Bio 4950, Undergraduate research, 3 credit hours, 2-3 Students, Fall and Winter semesters
2001-present:	Direct Immunology Journal Club, 1hour/week all year around, 40 student, postdocs and faculty members
1995-2001:	Microbiology 430 (Immunology), 3 credit hours, 45 lecture contact hours, 100-120 students, Fall semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Co-direct Microbiology 602 (Microbial Pathogenesis Journal Club), 1 credit hour, 15 lecture contact hours, 10-15 students, Fall semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Co-direct Microbiology 603 (Immunology Journal Club), 1 credit hours, 15 lecture contact hours, 10-15 students, Spring semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Microbiology 401 (Undergraduate Research), 3 credit hours, 1-2 students per semester, Microbiology, The University of Tennessee, Knoxville.

1998: Microbiology 630 (Topics in Immunology), 3 credit hours, 10 lecture contact hours, 20 students, Spring semester, (Seminar Series) Microbiology, The University of Tennessee, Knoxville.

1998-2001: Microbiology 493 (Independent Study in Immunology), 6 students, 10 lecture contact hours, spring, Microbiology, The University of Tennessee, Knoxville.

1992-1994: 600-level Immunology course, 3 credit hours, 6 lecture contact hours, 10 students, spring, Microbiology, Mount Sinai School of Medicine, New York.

HONORS AND AWARDS

2006. Speaker, Keystone Symposia on Tolerance Autoimmunity and Immune Regulation. March 21-26, 2006. Beaver Run Resort, Breckenridge, Colorado. Presentation title: Tregs for or against diabetes.

2004: Research Equipment Award for the purchase of an ELISPOT Analyzer, Office of Research, The University of Missouri,

2003: Keystone Symposia Scholarship ($1,000) for poster presentation by Hyun-Hee Lee, a graduate student in the laboratory, the meeting was held in Snowbird, UT

2003: Honorable citation for poster presentation by Randal Gregg, a graduate student in the laboratory. Life Science week, University of Missouri-Columbia.

2001: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

2000: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

2000: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of research Administration, The University of Tennessee, Knoxville.

1999: Chancellor's nomination for Howard Hughes Medical Institute Assistant Investigator Appointment, The University of Tennessee, Knoxville.

1999: Biological Equipment Award, Office of Research Administration/Science Alliance/Genome Science and Technology/Division of Biology, The University of Tennessee, Knoxville.

1999: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

1999: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of research Administration, The University of Tennessee, Knoxville.

1998: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

1998: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of Research Administration, The University of Tennessee, Knoxville.

1997: Biological Equipment Award, Office of Research Administration/Science Alliance/ Division of Biology/ Department of Microbiology, The University of Tennessee, Knoxville.

1997: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of Research Administration, The University of Tennessee, Knoxville.

1990: Research Excellence Award, Alliance Pharmaceutical Corporation. San Diego, CA.

1987-1988: Scientist Exchange Award (Postdoctoral Fellowship), French Cancer Society, Paris, France.

1984-1987: Graduate Student Scholarship, French Cancer Society, Paris, France.

PROFESSIONAL SERVICE

2007: Chair, Block symposium, regulation of immune cell development and function, American Association of Immunologis, Miami, FL.

2006-2010: Panel member, Hypersensitivity, Autoimmune and Immune-mediated Diseases (HAI) study section.

2006: Chair, Block symposium, treatment of autoimmune disease, American Association of Immunologis, Boston, MA.

2006: Review panel member, research proposals on Neurosciences, La Marato de TV3 Foundation, Catalan Agency For Health Technology Assessment And Research

2005: Chair, Block symposium, Cytokines and autoimmunity, American Association of Immunologis, Experimental Biology Meeting, San Diego, CA.

2004:	Panel member, NIAID Biodefence Workshop, Immunization and Vacination in Special Populations, Division of Allergy, Immunology and transplantation, NIH, Bethesda, MD
2004:	Chair, Block symposium, Tolerance and regulation of autoimmunity, American Association of Immunologis, Experimental Biology Meeting, Washington DC.
2004-present:	Adhoc Reviewer, TTT Study section, National Institutes of Health
2004-present:	Adhoc Reviewer, HAI Study section, National Institutes of Health
2003	Adhoc Reviewer, IMS Study Section, National Institutes of Health
2003	Adhoc Reviewer, ALY Study Section, National Institutes of Health
2003-present:	Member, Molecular Biology Program, University of Missouri-Columbia
2003-present:	Member, Genetics Area Program, University of Missouri-Columbia
2003-present:	Member, Veterinary Pathobiology Area Program, University of Missouri-Columbia
2003-present	Scientific Consultant, Division of endocrinology and Diabetes, Unversity of Missouri, Kansas City, MO
2002-2004:	Scientific Consultant, Alliance Pharmaceutical, San Diego, CA.
2001-present:	Member of The Graduate Student Recruitment Committee, Department of Molecular Microbiology and Immunology, The University of Missouri School of Medicine, Columbia.
2000-2001:	Adhoc Reviewer, BM-1 Study Section, National Institutes of Health
1992-2000:	Editorial board member: *Viral Immunology*
1989-present:	Reviewer: Immunology Journals
2000:	Guest Editor, International Review of Immunology
2000-2001:	Chair, Graduate Student Advisory Committee, Genome, Science, and Technology program, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.
1995-2001:	Member of The Graduate Student Recruitment Committee, Department of Microbiology, The University of Tennessee, Knoxville.
1998:	Member of Faculty Search Committee, Department of Comparative Medicine, College of Veterinary Medicine, The University of Tennesee, Knoxville.

1999: Panel Member: NIH/NCI, Small Business Innovation Research (SBIR)/Small Business Technology Transfer (STTR) Grant program. Flexible system to advance innovative research for cancer drug discovery by small business panel.

PROFESSIONAL MEMBERSHIP

2006-present: Member of the Henry Kunkel Society
1998-present: Member of the Society for Neuroscience
1992-present: Member of the American Association for the Advancement of Science.
1992-present: Member of the American Association of Immunologists.

PUBLICATIONS

Manuscripts published in peer-review journals

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APPENDIX B

Table 1. Blood Glucose Levels (mg/dl) for Treated Mice

	Mouse													
Week	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	161	165	180	182	165	250	179	212	160	229	232	224	180	173
1	252	125	134	159	144	137	129	179	152	258	183	154	165	199
2	176	119	213	127	144	136	169	165	146	162	227	192	152	148
3	121	117	145	121	117	255	142	228	281	285	217	186	112	111
4	149	110	127	148	126	179	124	151	390	176	214	156	159	132
5	131	144	175	117	116	153	126	128	351	98	99	161	140	136
6	148	132	114	136	150	126	123	143		94	134	97	118	156
7	98	99	146	133	93	105	121	156		89	120	179	135	134
8	128	111	178	152	113	158	119	139		170	127	172	134	108
9	109	104	140	110	120	138	153	147		147	134	142	116	132
10	118	108	160	138	120	140	121	141		145	170	132	117	151
11	151	91	192	144	101	145	113	152		143	112	114	135	163
12	107	91	244	161	130	151	109	216		150	124	149	121	97
13	107	101	256	124	113	137	108	184		142	114	130	148	137
14	85	81	264	125	116	112	119	155		154	127	154	118	143
15	133	113	198	96	120	118	99	156		147	119	178	123	158
16	136	91	285	112	128	103	112	127		153		144	146	157
17	111	129	377	105	111	148	107	134		228		123	123	111
18	127	99	366		98	158	113	148		350		159	122	93
19	99	110			111	176	137	338		339		170	132	
20	94	82			119	152	130	229				256	172	
21	83	96			114	135	153	331					215	
22	75	101				140	133	440						
23	70	99				140	154							
24	90	100				150	118							

Table 2. Blood Glucose Levels (mg/dl) for Untreated Mice

	Mouse						
Week	11	12	13	14	15	16	17
0	174	169	169	168	175	199	219
1	293	366	155	159	251	240	379
2	352	400	157	200	340	450	400
3	457	-	200	249	450	-	-
4	-	-	270	393	-	-	-
5	-	-	376	400	-	-	-
6	-	-	400	-	-	-	-

Figure 1

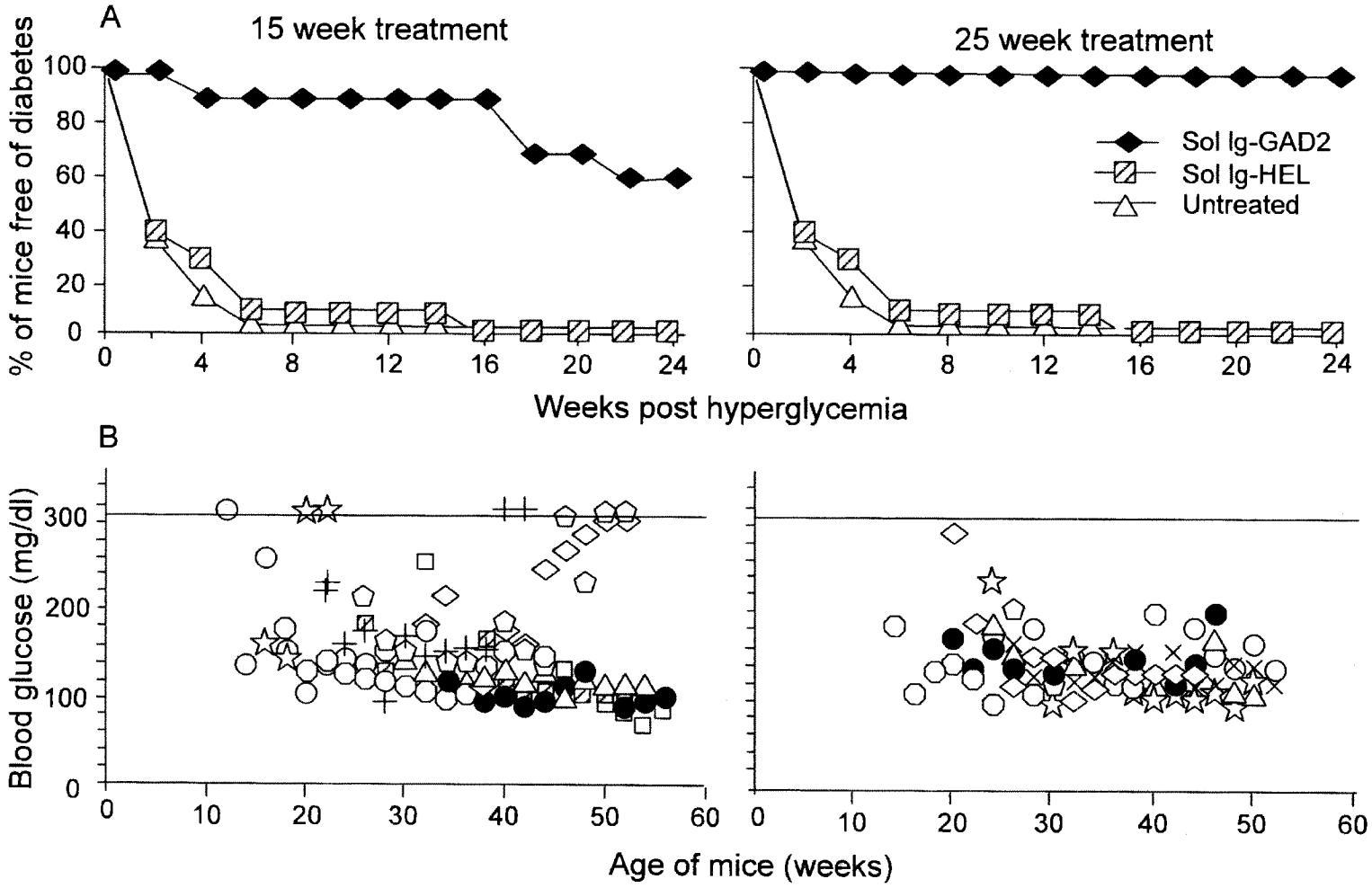
A
15 week treatment
25 week treatment
% of mice free of diabetes
100
80
60
40
20
0
0
4
8
12
16
20
24
Sol Ig-GAD2
Sol Ig-HEL
Untreated
Weeks post hyperglycemia
B
Blood glucose (mg/dl)
300
200
100
0
0
10
20
30
40
50
60
Age of mice (weeks)

EXHIBIT Q

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ARTICLE

Innocuous IFNγ induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production

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The role of Th17 cells in type I diabetes (TID) remains largely unknown. Glutamic acid decarboxylase (GAD) sequence 206–220 (designated GAD2) represents a late-stage epitope, but GAD2-specific T cell receptor transgenic T cells producing interferon γ (IFNγ) protect against passive TID. Because IFNγ is known to inhibit Th17 cells, effective presentation of GAD2 peptide under noninflammatory conditions may protect against TID at advanced disease stages. To test this premise, GAD2 was genetically incorporated into an immunoglobulin (Ig) molecule to magnify tolerance, and the resulting Ig-GAD2 was tested against TID at different stages of the disease. The findings indicated that Ig-GAD2 could not prevent TID at the preinsulitis phase, but delayed TID at the insulitis stage. More importantly, Ig-GAD2 sustained both clearance of pancreatic cell infiltration and β-cell division and restored normoglycemia when given to hyperglycemic mice at the prediabetic stage. This was dependent on the induction of splenic IFNγ that inhibited interleukin (IL)-17 production. In fact, neutralization of IFNγ led to a significant increase in the frequency of Th17 cells, and the treatment became nonprotective. Thus, IFNγ induced by an adjuvant free antigen, contrary to its usual inflammatory function, restores normoglycemia, most likely by localized bystander suppression of pathogenic IL-17–producing cells.

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Abbreviations used: GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; IAA, insulin autoantibody; TID, type I diabetes.

Antigen-specific approaches have been defined that could prevent the development of type I diabetes (TID; for review see [1]). However, antigen-driven strategies that could counter the disease at more advanced stages have yet to be defined (1). As with many autoimmune disorders, TID most likely involves multiple autoantigens and diverse T cell specificities (2, 3). In addition, sequential spreading seems to orchestrate TID, with insulin being required for the initiation of the disease (4), whereas GAD-reactive T lymphocytes are more involved at later stages of TID (5, 6). Thus, for an antigen-specific therapy to be effective and practical against TID, it would have to target late-stage epitopes that could counter diverse aggressive T cell specificities. GAD2 peptide corresponding to amino acid sequence 206–220 of GAD is considered a late-stage epitope because its T cell reactivity is detected at an advanced stage of the disease (7). TCR transgenic T cells specific for GAD2 peptide were generated, but these produced both IFNγ and IL-10 and were protective against TID when tested in a transfer model of passive diabetes (8). Given this information, we reasoned that effective presentation of GAD2 peptide in vivo under noninflammatory conditions would possibly induce IFNγ- and IL-10–producing T cells that could protect against TID. Because IFNγ displays inhibitory activity against Th17 cells (9, 10), the approach could prove effective even at an advanced stage of the disease if Th17 cells play a pathogenic role in TID. To test these premises, GAD2 peptide was genetically inserted into the variable region of a heavy chain Ig gene, and the fusion gene was transfected into

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a myeloma B cell line along with the parental light chain gene for expression as a complete Ig-GAD2. Because Igs internalize into APCs via Fcγ receptor (FcγR), the grafted GAD2 peptide will be efficiently dragged into the cells, where it accesses newly synthesized MHC class II molecules, and presentation will be significantly increased relative to free peptide, as was the case for other diabetogenic and encephalitogenic peptides (11–16). Moreover, because Igs are self-proteins, when injected into animals, presentation occurs without inflammation, leading to lack of costimulation and magnification of tolerance (12–14).

In an initial attempt, Ig-GAD2 was tested for prevention of TID before insulitis, but proved ineffective for delay of disease. However, when the treatment was administered at the insulitis stage, protection against TID was observed. More importantly, Ig-GAD2 given to hyperglycemic mice at the prediabetic stage was highly effective, leading to clearance of pancreatic cell infiltration, stimulation of β-cell division, and restoration of normoglycemia. Investigation of the mechanism underlying reversal of disease revealed the presence of splenic IFNγ-producing GAD2-specific T cells that were, indeed, responsible for reversal of disease because neutralization of IFNγ restored progression to overt diabetes. In parallel, the protected mice had reduced production of IL-17 cells in the spleen and pancreas relative to diabetic mice, and exogenous IL-17 reinstated progression to diabetes in the otherwise protected animals. Thus, splenic IFNγ likely interferes with supply of Th17 to the pancreas, leading to clearance of islet infiltration, stimulation of β cell division, and restoration of normoglycemia.

RESULTS

Treatment with Ig-GAD2 restores normoglycemia

The I-A^{g7}–restricted diabetogenic GAD2 peptide was genetically expressed on an Ig molecule, and the resulting Ig-GAD2 was used to test against TID. Similarly, the nondiabetogenic I-A^{g7}–restricted hen egg lysozyme (HEL) 11–25 sequence was also incorporated in an Ig, and the resulting Ig-HEL was used as control (16). The chimeras were then tested for presentation to a GAD2-specific T cell line. As indicated in Fig. 1, Ig-GAD2 was taken up by APCs, processed, and presented to GAD2-specific T cells much more efficiently than free GAD2 peptide (Fig. 1 A, B). The control Ig-HEL was unable to induce similar stimulation of the GAD2-specific T cells. Ig-GAD2 was then assayed for tolerogenic function by testing for prevention of TID in young NOD mice undergoing the initial phase of islet infiltration, which is referred to as the preinsulitis stage. The results in Fig. 1 C indicate that Ig-GAD2 had no significant long-term protective effect against TID relative to Ig-HEL or untreated mice. Knowing that insulin, but not GAD, is required for initiation of diabetes at the preinsulitis stage (4), the lack of protection might have been caused by the absence of activated GAD2-specific target T cells at this stage. We then tested Ig-GAD2 for suppression of diabetes at a later stage during insulitis. It has been shown that seroconversion to insulin autoantibody (IAA) production is indicative of ongoing insulitis (17, 18), and our own studies indicated that among the 83% of female NOD mice that seroconvert to IAA at the age of 8–11 wk, 84% develop overt

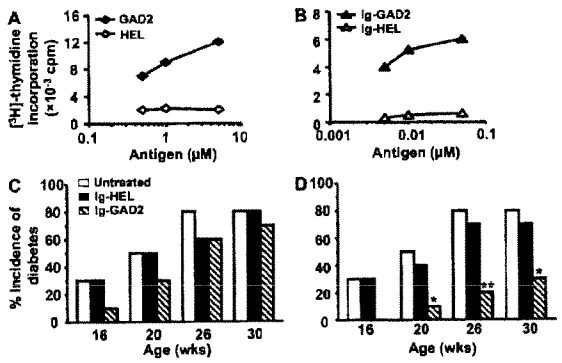

Figure 1. Ig-GAD2 treatment given at the insulitis-positive stage reverses T1D. (A and B) Presentation of Ig-GAD2 to T cells. NOD splenic APCs were incubated with free peptides (A) or Ig chimeras (B), and 1 h later GAD2-specific T cells were added. Activation was assessed by [³H]thymidine incorporation. HEL peptide and Ig-HEL were included as negative controls. (C and D) Percentage of mice free of diabetes upon treatment with Ig-GAD2 or the control Ig-HEL at the preinsulitis (C) and insulitis (D) stage, respectively. All mice were monitored for blood glucose from 12 to 30 wk of age. *, $P < 0.05$; **, $P < 0.01$ compared with untreated mice. A mouse is considered diabetic when blood glucose level is ≥ 300 mg/dl for two consecutive weeks. An untreated group was included in all experiments for comparison purposes. At least 10 mice were included in each experimental group.

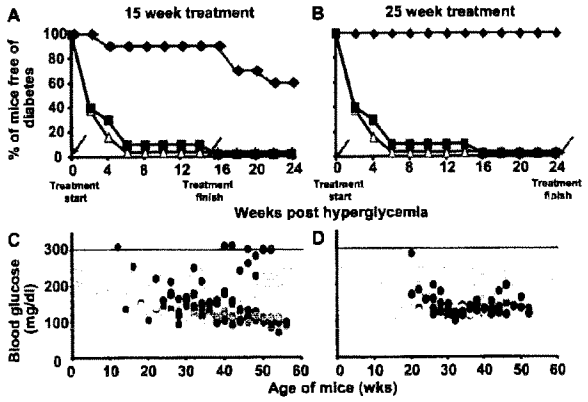

Figure 2. Ig-GAD2 treatment given at the prediabetic stage reverses T1D. (A and B) Percentage of mice free of diabetes upon treatment with Ig-GAD2 or the control Ig-HEL at the hyperglycemic stage for 15 (A) or 25 wk (B). Arrows indicate the beginning and end of treatment. (C and D) Individual blood glucose levels of Ig-GAD2 treated mice are shown from the week of diagnosis of hyperglycemia up to 52 or 56 wk of age for 15 (C) and 25 wk (D) treatment regimens. Each dot represents a different mouse. A mouse is considered hyperglycemic or diabetic when blood glucose level is between 160–250 or ≥ 300 mg/dl for two consecutive weeks, respectively. The shaded area indicates the hyperglycemic range of blood glucose levels and the line depicts the diabetic level. An untreated group was included in all experiments for comparison purposes. At least 10 mice were included in each experimental group.

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diabetes (16). Ig-GAD2 was then tested for delay of T1D upon IAA-seroconversion. An initial regimen consisting of 300 μg of Ig-GAD2 at week 1, 2, and 3 upon IAA seroconversion indicated that 50% of mice were protected against diabetes up to 30 wk of age (unpublished data). This was promising, as the same regimen did not protect at the preinsulitis stage, and it prompted us to test a prolonged regimen for suppression of diabetes. As indicated in Fig. 1 D, administration of Ig-GAD2 into insulitis-positive (IAA^+) mice delayed T1D, and most of the animals (7 out of 10) remained free of disease by week 30 of age. Ig-HEL–treated animals, like the untreated group, were not significantly protected (Fig. 1 D). These results indicate that Ig-GAD2 protects against T1D at a later, rather than earlier, stage of the disease. We then evaluated Ig-GAD2 at the more advanced hyperglycemic stage. Accordingly, blood glucose levels were monitored beginning at week 12 of age, and mice displaying hyperglycemia between the ages of 14 to 30 wk were subjected to a daily injection of Ig-GAD2 for 5 d, and then a weekly injection for either 15 or 25 wk. The results show that 90% of the mice under the 15-wk Ig-GAD2 regimen were protected against diabetes throughout the 15 wk of treatment (Fig. 2 A). However, only 60% of the mice remained disease-free for the 10 wk after cessation of treatment. Untreated and Ig-HEL recipient mice became diabetic by the fifth week of hyperglycemia. When the regimen was extended to 25 wk, 100% of the Ig-GAD2–treated animals were protected (Fig. 2 B), and normoglycemia was restored in all mice. This status persisted throughout the duration of the study (mice aged 52–56 wk). The weekly blood glucose level of individual mice shows a consistent pattern of return to normoglycemia for 6 out of 10 mice in the 15-wk treatment regimen, and all 10 animals in the 25-wk regimen (Fig. 2, C and D). A detailed description of the day of onset, as well as the level of blood glucose at the beginning and termination of the hyperglycemic treatment regimen, is provided in Table I. These results demonstrate that protection against the disease by Ig-GAD2 occurs at the onset of insulitis, whether this manifests at an early or an older age. Overall, this antigen-specific single-epitope therapy by Ig-GAD2 restores normoglycemia in prediabetic mice, a stage at which GAD2-specific T cells could be targeted.

Treatment with Ig-GAD2 increases the number of healthy pancreatic islets

To determine whether the restoration of normoglycemia by Ig-GAD2 is caused by interference with cell infiltration, the mice were subjected to histopathologic analysis upon

Table I. Blood glucose (BG) level at the onset of hyperglycemia and at termination of treatment regimen[a]

Mouse ID	Age at onset of hyperglycemia (wk)	Blood glucose level before treatment (mg/dl)[b]	Blood glucose level after termination of treatment (mg/dl)[c]
15-wk treatment regimen			
106.1	30	161	133
106.7	30	165	113
119.2	30	180	285[d]
119.8	26	182	112
119.9	28	165	128
190.1	12	250	118
191.2	18	179	99
192.2	26	212	134[d]
192.3	16	160	308[d]
196.1	22	222	150[d]
25-wk treatment schedule			
220.1	26	160	135
221.12	24	174	120
206.2	24	231	121
203.1	24	181	110
232.3	20	173	140
236.6	14	185	116
225.9	26	163	120
237.8	20	250	130
227.7	28	180	137
244.4	16	171	123

[a]The results illustrated in this table were from the mice treated with either the 15- or 25-wk Ig-GAD2 treatment regimen and described in Fig. 2. The hyperglycemia onset represent the week during which the mice showed, for the first time, a blood glucose level of 160–250 mg/dl.
[b]Blood glucose level obtained the second week of hyperglycemia.
[c]Blood glucose level obtained at the termination of treatment regimen.
[d]Mice that became diabetic before or within 7 wk of treatment termination.

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completion of the treatment regimen. As indicated in Fig. 3, most of the islets in hyperglycemic and diabetic mice exhibited intrainsulitis (Fig. 3 A, 1, 2, and 3), the majority of islets in treated mice were not inflamed (Fig. 3 A, 4) or had only mild periinsulitis (Fig. 3 A, 5 and 6). Moreover, enumeration of the islets indicated that the treated animals had a significantly greater number of total islets than the hyperglycemic or diabetic mice (Fig. 3 B). The number of insulin-positive islets also increased from 14 per pancreas at the prediabetic (hyperglycemic) stage to 29 per pancreas upon treatment with Ig-GAD2. Also, the 15-wk group had a higher number of islets with periinsulitis (38 vs. 30%) or no insulitis (35 vs. 17%) relative to the hyperglycemic stage (Fig. 3 C). On the other hand, the number of islets with severe and mild intrainsulitis were reduced in the treated versus hyperglycemic mice (8 and 19% vs. 22 and 31%, respectively). Surprisingly, in the 25-wk treatment group, although the total number of islets was reduced to that of the hyperglycemic stage, most of these islets exhibited no insulitis (60%), periinsulitis (28%), or mild intrainsulitis (12%; Fig. 3 C). Overall, the treatment with Ig-GAD2 led to a significant increase in the number of noninflamed (healthy) islets.

Ig-GAD2–treated mice exhibit pancreatic β-cell division

The increase in the number of healthy islets in the treated mice could be caused by regression of cell infiltration and/or formation of new β cells. To address this premise, the treated mice were injected with the proliferation indicator BrdU and killed, and pancreatic sections were double stained with anti-insulin and -BrdU antibodies and analyzed for BrdU incorporation

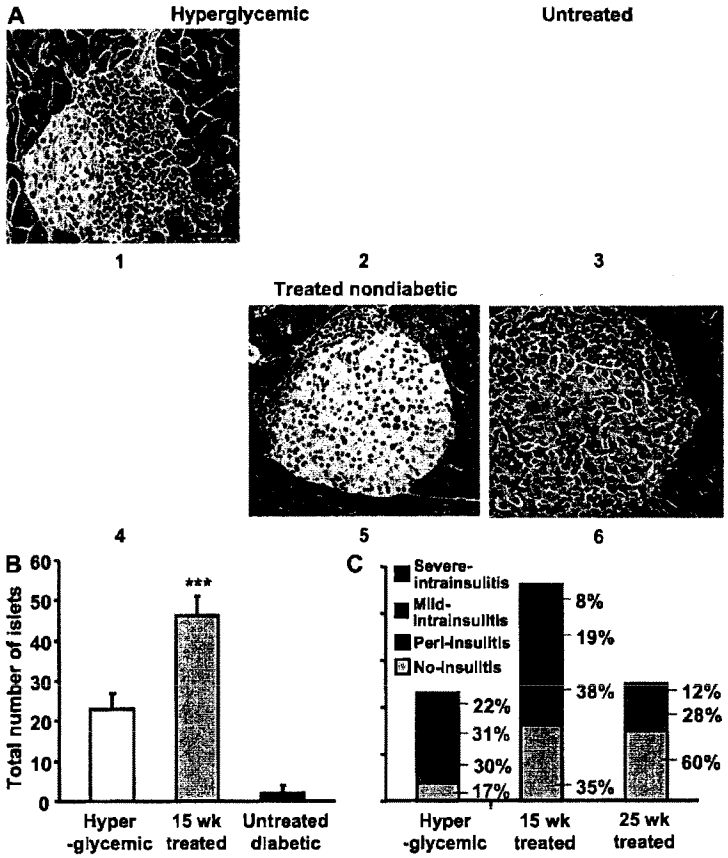

Figure 3. Ig-GAD2 treatment diminishes insulitis and increases the total number of islets. (A) Pancreatic histology. Four sections per pancreas (8 μm thick each cut 100 μm apart) from 5 hyperglycemic (A, 1 and 2), untreated diabetic (A, 3) or 15-wk Ig-GAD2–treated nondiabetic (A, 4, 5, and 6) mice were stained with hematoxylin and eosin and analyzed at 400× magnification. For the hyperglycemic and untreated diabetic mice sections were made the second week of diagnosis. For the treated nondiabetic mice, histology was performed 7 wk after the last treatment. (B) Total islets per pancreas as determined by hematoxylin and eosin staining from the three groups of mice described in A. Only structures with visible islet cells and incomplete infiltration were counted. (C) Islets from hyperglycemic, 15 and 25 wk Ig-GAD2–treated mice were scored as described in Materials and methods, and the percentages represent the number of islets of a given score over the total number of islets from B. The sections were made at the time indicated in A, and 2 d after the last Ig-GAD2 injection for the 25 wk–treated group. ***, $P = 0.0001$ for the total number of islets in 15 wk–treated versus hyperglycemic group. Error bars indicate the SD. Bars, 25 μm.

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and insulin production. BrdU staining was visible in the highly proliferative luminal intestinal cells used as control, but these had no staining with anti-insulin antibody (Fig. 4 A). Islets of nondiabetic 5-wk-old mice were positive for insulin, but did not incorporate BrdU, suggesting that these insulin-producing β cells were not dividing (Fig. 4 B). Thus, under normal circumstances, insulin production emanates from existing β cells whose nuclei do not incorporate BrdU, giving a minimal number of BrdU/insulin double-positive ($BrdU^+/insulin^+$) β cells (Fig. 4 E). The hyperglycemic mice showed very few insulin-producing β cells and no BrdU incorporation (Fig. 4 C), resulting in an insignificant number of $BrdU^+/insulin^+$ cells (Fig. 4 E). In contrast, islets from the 25-wk treatment group showed $insulin^+$ β cells that were either $BrdU^-$ (residual β cells) or $BrdU^+$ (newly formed β cells; Fig. 4 D). Notably, the number of these insulin-producing dividing β cells was significantly increased in all 10 mice in which treatment restored normoglycemia (Fig. 4 E). Interestingly, the total number of dividing cells producing insulin ($BrdU^+/insulin^+$) was low, and it may not solely account for the restoration of normoglycemia. $BrdU^-/insulin^+$ residual islet cells, which amounted to 81 cells per pancreas, may have also contributed to the control of blood glucose levels, and these likely represent a combination of newly formed and residual β cells that were rescued by regression of infiltration. There was a minimal number of dividing β cells ($BrdU^+/insulin^+$) in the normal and hyperglycemic groups, despite the presence of 927 and 50 $BrdU^-/insulin^+$ β cells, respectively. These results indicate that treatment with Ig-GAD2 reduces cell infiltration, leading to rescue of residual and formation of new β cells.

Ig-GAD2–treated mice produce protective IFNγ

Previous studies indicated that Ig-GAD1, which is an Ig chimera carrying GAD524-543, and Ig-INSβ carrying insulin 9–23 aa residues induce T regulatory (T reg) cells and prevent T1D only when given in an aggregated, but not soluble, form (15, 16). This is because aggregated, but not soluble, Ig chimeras cross-link FcγR on APCs, induce IL-10 by the presenting cells, and expand T reg cells (15, 16). In this study, only soluble Ig-GAD2 was used for treatment. Despite the fact that soluble Ig-GAD2 does not induce the production by APCs of the T reg cell growth factor IL-10 (19) and is predicted not to expand T reg cells, it was tested for expansion of T reg cells in hyperglycemic mice before and after treatment with Ig-GAD2. The results indicated that the percentage of $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+FoxP3^+$ T cells in the spleen, as well as in the pancreatic lymph nodes, were similar before and after treatment (Table II). This suggests that T reg cells play a minimal role in disease reversal by soluble Ig-GAD2.

For Ig-GAD2 therapy, it is logical to contemplate that the resolution of the inflammatory infiltration is caused by modulation of GAD2-reactive diabetogenic T cells. Because T1D is likely to involve multiple autoantigens, the restoration of normoglycemia would require modulation of diverse T cell specificities. Thus, the plausible hypothesis postulates that

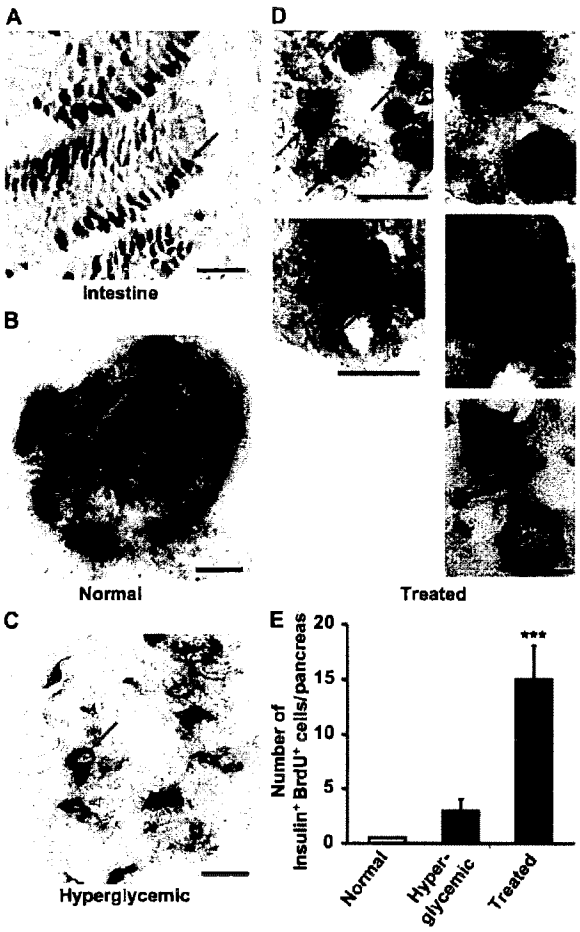

Figure 4. β Cells from mice treated with Ig-GAD2 incorporate BrdU. Mice (10 per group) were given 100 mg/kg BrdU i.p. and killed 3 h later. Sections of the small intestine or pancreas were stained with anti-insulin and -BrdU antibodies, and then analyzed for insulin production (blue cytoplasmic rim) and BrdU incorporation (red nuclei) at 400× magnification. Blue arrows indicate $BrdU^+$ cells, green arrows indicate $insulin^+$ cells, and red arrows indicate $BrdU^+/insulin^+$ cells. Intestinal lumen (A) and β cells (D) from mice recipient of the 25-wk Ig-GAD2 regimen. (B) Beta cells from 5-wk-old nondiabetic NOD mice. (C) β Cells from hyperglycemic mice. (E) Total number of $insulin^+/BrdU^+$ cells in nondiabetic (normal), hyperglycemic, and Ig-GAD2–treated nondiabetic NOD mice. ***, $P = 0.0001$, treated group compared with hyperglycemic group. Error bars indicate the SD of 10 pancreata. Bars: (A–C) 25 μm; (D, left) 20 μm; (D, right) 5 μm D.

recovery from the disease involved localized bystander suppression. To test this premise, the splenic cells from recovered mice were stimulated with GAD2 peptide and assessed for both suppressive and inflammatory cytokines. The results indicate that although no measurable IL-4 or TGFβ was detected (not depicted), there was significant IFNγ and IL-10 production by these cells relative to the control HEL peptide (Fig. 5 A). Moreover, intracellular cytokine analysis of CD4 and Vβ8.2

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Table II. Reversal of T1D by Ig-GAD2 does not significantly increase expression of phenotypic markers associated with T reg cells[a]

	$CD4^+CD25^+FoxP3^+$		$CD4^+CD25^+CD62L^+$	
	Untreated	Treated	Untreated	Treated
SP	4.9	3.3	2.0	1.9
PLN	5.0	5.5	3.0	4.4

[a]Spleen and pancreatic lymph node cells from Ig-GAD2–treated and control untreated mice were stained with anti-CD4, -CD25, and -CD62L or -FoxP3 antibody. The cells were gated on $CD4^+$ cells and analyzed for CD25, FoxP3, and CD62L expression by flow cytometry.

T cells indicated that the majority of the T cells produced only IFNγ, with fewer cells stained positive for both IL-10 and IFNγ (Fig. 5 B). Indeed, upon stimulation with GAD2 peptide, a significant increase (four- to sevenfold) in the number of CD4/Vβ8.2 T cells producing IFNγ was observed in the Ig-GAD2–treated versus untreated mice. Because IL-10 is known for its anti-Th1 suppressive function (20–22), we suspected that protection against the disease involves the function of these IL-10/IFNγ–producing cells. To our surprise, however, when in vivo cytokine neutralization was performed along with Ig-GAD2 treatment, the recovery persisted with anti–IL-10 treatment, but was nullified by removal of IFNγ (Fig. 5 C). Isotype-matched rat IgG had no effect on the disease (Fig. 5 C). These observations indicate that IFNγ, contrary to its well-defined inflammatory function, is likely involved in modulation of inflammation and restoration of normoglycemia.

Ig-GAD2 treatment interferes with IL-17 production in an IFNγ-dependent fashion

Th17 cells represent a newly defined subset of pathogenic T cells whose development can be facilitated by TGFβ and

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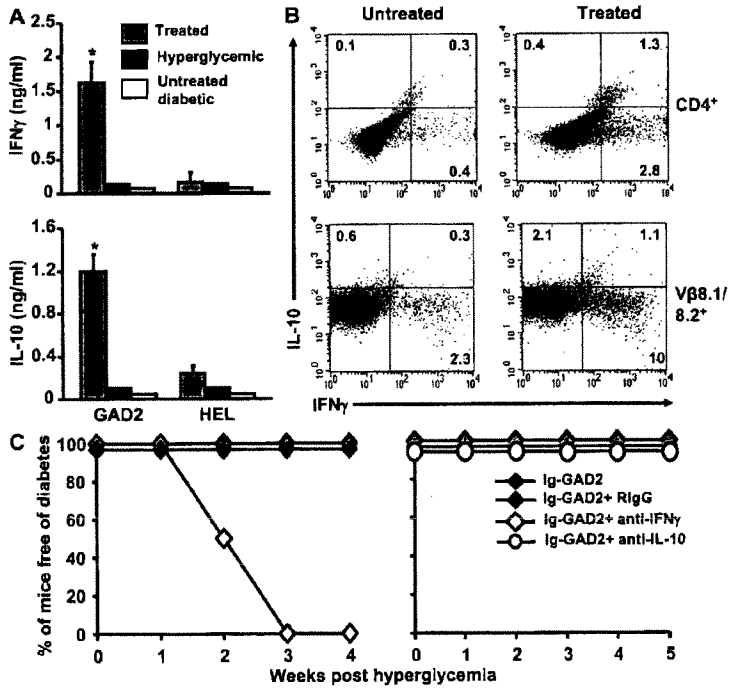

Figure 5. Treatment with Ig-GAD2 induces IFNγ that sustains protection against diabetes. (A) Splenocytes from hyperglycemic mice recipient of the 25-wk Ig-GAD2 treatment regimen were stimulated in vitro with GAD2 and the control HEL peptide and IFNγ and IL-10 were measured by ELISA as described in Methods. Diabetic as well as untreated hyperglycemic mice were included for control purposes. Each bar represents the mean ± SD of three independent experiments. *, P = 0.01 when stimulation by GAD2 is compared with HEL peptide. (B) Intracellular IL-10 and IFNγ production by splenic CD4 (top) or Vβ8.1/8.2 (bottom) T cells from the 25-wk–treated (right) and hyperglycemic untreated (left) mice. This was done by intracellular staining upon stimulation with GAD2 peptide, as indicated in the Materials and methods. Data are representative of three independent experiments. (C) Percentage of mice free of diabetes upon in vivo neutralization of IFNγ (left) or IL-10 (right) during treatment with Ig-GAD2 at the hyperglycemic stage. Anti-IFNγ (R4-6A4), anti-IL-10 (JES5-2A5), or isotype control rat IgG were given to mice (500 μg/mouse per injection) i.p. every 3 d for 4 consecutive weeks, beginning with the first injection of Ig chimeras. The mice received a total of nine antibody injections. At least eight mice were included in each experimental group.

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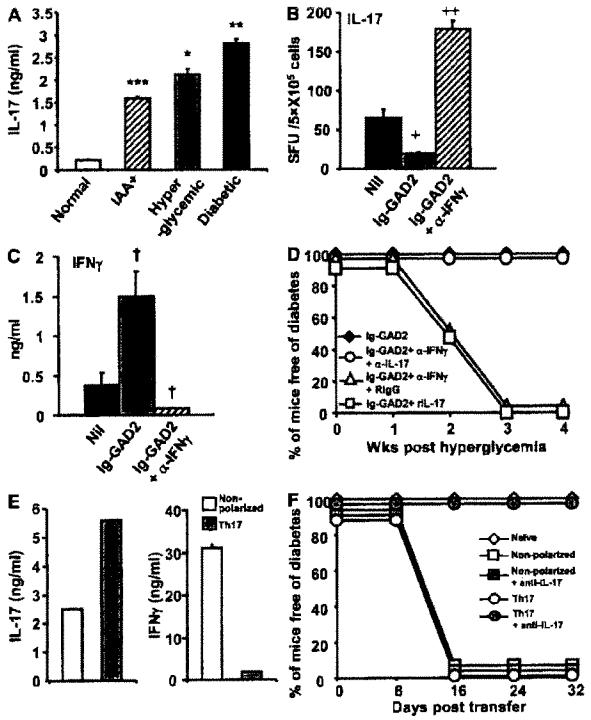

Figure 6. Neutralization of IFNγ during treatment with Ig-GAD2 restores IL-17 production. (A) IL-17 response from the splenocytes of preinsulitis (normal), insulitis-positive (IAA+), hyperglycemic, and diabetic mice upon in vitro stimulation with anti-CD3 antibody. Data are representative of three independent experiments. ***, P = 0.0004, insulitis-positive versus normal; *, P = 0.02, hyperglycemic versus insulitis-positive; **, P = 0.005, diabetic versus hyperglycemic group. IL-17 (B) and IFNγ (C) response from splenocytes of mice recipient of anti-IFNγ during treatment with Ig-GAD2 at the hyperglycemic stage. Splenocytes were harvested when the mice became diabetic on the fourth week of treatment; they were stimulated in vitro with GAD2 peptide, and their responses were measured by ELISPOT and ELISA, respectively. Nil (diabetic) and Ig-GAD2-treated groups were included as controls. Data are representative of three independent experiments. †, P = 0.01, treated versus nil group; ††, P = 0.001, Ig-GAD2 + anti-IFNγ versus Ig-GAD2 group. †, P = 0.04, Ig-GAD2 versus nil group; †, P = 0.02, Ig-GAD2 + anti-IFNγ versus Ig-GAD2 group. (D) Percentage of mice free of diabetes upon administration of recombinant IL-17 or neutralization of both IFNγ and IL-17 during treatment with Ig-GAD2 at the hyperglycemic stage. IL-17 was administered (1 μg/mouse per injection) i.p. daily for 5 consecutive days, beginning with the first injection of Ig-GAD2. Subsequently, the mice received an injection of rIL-17 every week, along with Ig-GAD2. An injection of anti-IFNγ (R4-6A4; 500 μg/mouse) and anti-IL-17 (TC11-18H10; 200 μg/mouse) was given on the first day of treatment with Ig-GAD2 after diagnosis of hyperglycemia. Four additional injections were given at 4-d intervals. At least eight mice were included in each experimental group. (E and F) Th17-polarized cells induce diabetes. (E) IL-17 (left) and IFNγ (right) responses from the nonpolarized and Th17 polarized splenocytes were measured by ELISA. Each bar represents the mean ± SD of triplicate wells. (F) Percentage of mice free of diabetes upon adoptive transfer of 10×10^6 naive, nonpolarized and Th17-polarized cells in NOD.scids (4–6 wk old). Additional groups received IL-17 neutralizing antibody, along with Th17-polarized and nonpolarized cells for control purposes. Anti–IL-17 antibody (TC11-18H10; 200 μg/mouse) was given on the day of transfer, and two additional injections were given at day 4 and 16 after transfer.

IL-6 or interfered with by IFNγ or IL-27 (9, 10, 23–26). Because Ig-GAD2 treatment induces IFNγ, we sought to determine whether restoration of normoglycemia involves interference with IL-17 production. Accordingly, we began by assessing whether IL-17 is produced by NOD T cells, and followed the pattern of its secretion during disease progression. Fig. 6 A shows that stimulation with anti-CD3 antibody did not induce measurable IL-17 by splenocytes from normal 4-wk old mice. However, IL-17 was evident upon IAA-seroconversion and increased significantly when the mice progressed to hyperglycemia and diabetes. The treatment with Ig-GAD2 at the hyperglycemic stage significantly reduced the frequency of GAD2-specific IL-17–producing cells as measured by spot formation (Fig. 6 B). However, neutralization of IFNγ by administration of anti-IFNγ antibody along with Ig-GAD2 restored even higher frequency of Th17 cells. This Th17 restoration is likely caused by complete neutralization of IFNγ because IFNγ-producing Th1 cells could not be detected by ELISPOT (not depicted) and no measurable IFNγ cytokine was found by ELISA (Fig. 6 C). It is thus likely that the restoration of diabetes by neutralization of IFNγ during treatment with Ig-GAD2 (Fig. 5 C) is caused by restoration of Th17. In fact, administration of rIL-17 along with Ig-GAD2 treatment nullifies tolerance and restores diabetes (Fig. 6 D). Moreover, administration of both anti-IFNγ and –IL-17, but not anti-IFNγ and rat IgG, simultaneously protects against T1D (Fig. 6 D), further confirming the interplay between IFNγ and IL-17. To ensure that Th17 cells can be diabetogenic, we chose the BDC2.5 TCR transgenic T cells (27) for polarization with anti-CD3 and -CD28 antibodies and tested for transfer of diabetes into NOD.scid mice. The rationale for this choice instead of Ig-GAD2–induced Th17 cells lies in the fact that the BDC2.5 cells are well characterized and represent a homogeneous population in which the number of cells to be transferred can be precisely controlled. In addition, the Ig-GAD2/NOD model represents a polyclonal system in which the different subsets of T cells cannot be separated. Thus, BDC2.5 T cells were stimulated with anti-CD3 and -CD28 antibodies in the presence or absence of Th17 polarizing factors, and the cells were tested for transfer of diabetes into NOD.scid mice.

As indicated in Fig. 6 E, the Th17-polarized cells had enhanced levels of IL-17 compared with nonpolarized cells, but no measurable IFNγ, whereas nonpolarized cells produced significant IFNγ. These results indicate that the polarization to Th17 was significant under the chosen conditions. Furthermore, when the polarized cells were transferred into NOD.scid mice, diabetes manifested within 16 d after transfer, as with activated, but not polarized, T cells (Fig. 6 F). Diabetes did not occur when the transfer was made with naive BDC2.5 cells. In addition, when IL-17 was neutralized by injection of anti–IL-17

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antibody in the mice recipient of Th17-polarized cells, the disease did not manifest. However, neutralization of IL-17 did not protect against diabetes transferred by nonpolarized cells. These results indicate that Th17 cells producing IL-17 can transfer diabetes into naive mice. The results are thus interpreted to indicate that Ig-GAD2 mobilizes IFNγ-producing splenic Th1 cells that interfere with IL-17–producing diabetogenic lymphocytes to reduce inflammation, sustain islet formation, and restore normoglycemia.

Treatment with Ig-GAD2 sustains long-lasting production of IFNγ in the spleen and nullifies IL-17 in the pancreas

At the hyperglycemic stage, most of the pathogenic T cells likely reside in the pancreas as differentiated cells that have already been exposed to antigen (28, 29). Because IFNγ has been suggested to interfere with the differentiation of naive cells into Th17 (9, 10), it is likely that IFNγ Th1 cells operate their interference with Th17 in the spleen or pancreatic lymph nodes rather than the pancreas. Analysis of the dynamics of both populations at the beginning, as well as at the end, of Ig-GAD2 treatment indicated that during the initial phase of hyperglycemia, IFNγ-producing Th1 cells are mostly located in the spleen (Fig. 7 A), whereas Th17 cells reside in the pancreas (Fig. 7 B). However, at the end of the treatment, Th1 cells remain in the spleen (Fig. 7 C), whereas Th17 cells are undetectable in any organ (Fig. 7 D). These results suggest that Ig-GAD2 induces IFNγ in the spleen, which likely interfere with differentiation of naive cells into Th17 cells, resulting in a diminished supply of these cells to the pancreas.

DISCUSSION

Treatment with anti-CD3 antibody alone has been shown to reverse diabetes, but disease reoccurrence has been observed (30–32). This justifies the search for new strategies, and the combination of anti-CD3 antibody with antigen-specific therapy did overcome rebounding of disease (33). Because T1D involves multiple autoantigens that likely manifest their activities at different stages of the disease, it has been difficult to define antigen-based regimens that could reverse the disease process at an advanced stage (1). Because Igs have proven powerful for enhancing tolerogenic function of peptides (12, 16) and GAD2 peptide was defined as a late-stage protective epitope (5–8), we incorporated GAD2 peptide onto an Ig molecule and tested the resulting Ig-GAD2 for protection, as well as reversal of advanced T1D process. Surprisingly, Ig-GAD2 was not protective at the preinsulitis stage (Fig. 1 C), but delayed the disease when it was administered upon IAA seroconversion (Fig. 1 D) (14). This differential efficacy may be related to delayed spreading of GAD2-specific T cells that become available for targeting at an advanced stage of the disease (7). This has prompted us to test for reversal of T1D at the prediabetic stage, when blood glucose has reached an abnormal level. Again, Ig-GAD2 was able to restore long-lasting normoglycemia in most of the animals (Fig. 2, A and C), and when the regimen was extended to 25 wk (Fig. 2, B and D) all mice were protected. To date, we have tested >30 mice with

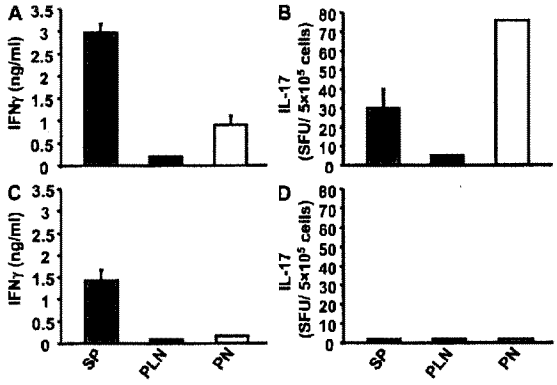

Figure 7. Splenic IFNγ induced by Ig-GAD2 treatment diminishes splenic and pancreatic IL-17 causing reversal of diabetes. IFNγ and IL-17 cytokine responses of splenic, pancreatic, and pancreatic lymph node cells from mice treated with Ig-GAD2 for 1 wk (A and B) or 25 wk (C and D) starting from the week of hyperglycemia diagnosis. The cells were stimulated with GAD2 peptide, and the responses were measured by ELISA for IFNγ and ELISPOT for IL-17, as indicated in Materials and methods. Each bar represents the mean ± SD of two independent experiments.

the 25-wk regimen and all animals maintained normoglycemia until they were terminated at 52 or 56 wk of age for other use. At the histology level, the 15-wk regimen reduced infiltration, and this was accompanied by an increase in the total number of islets relative to the beginning of the treatment (Fig. 3 B). However, with the 25-wk regimen, the number of islets dropped back to where it was at the hyperglycemic stage, but the majority of these were healthy islets. We believe that both eradication of infiltration from slightly infiltrated islets as well as formation of new islets were part of the repair process. The initial increase and the return of the number of islets may reflect dynamics in which new islets were formed while severely infiltrated islets were eliminated and those mildly infiltrated were cleaned up. This is drawn from Fig. 3 C, which illustrates the evolution of status and number of islets during the treatment and is supported by the BrdU incorporation observed with insulin-producing β cells (Fig. 4). There was, in fact, a substantial increase of dividing cells that were simultaneously producing insulin, in addition to cells that were producing insulin without evidence of division (Fig. 4). The formation of new islets has been reported before, but whether these are the product of stem cells maturation, origination from splenic cells, or division of residual β cells remains unknown (34–38). It has been shown that physical damage of islets stimulates β cell division (38). In Ig-GAD2–treated mice, upon clearance of inflammatory cells the damaged islets may likewise spontaneously initiate division of β cells. However, given that exogenous compounds such as CFA (35–37) and now Ig-GAD2 allow for β cell division, it may be that clearance of inflammatory cells minimizes cell division inhibitory factors, leading to proliferation of residual β cells or differentiation of β cell progenitors. The precise mechanism underlying β cell proliferation is of great interest, and

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effort is being made to determine how Ig-GAD2 leads to β cell division. What is important here is that a single-epitope treatment could lead to eradication of infiltration involving diverse T cell specificities. One has to assume that there must be at least a local bystander suppression that targets GAD2-specific T cells and unrelated neighboring cells. When the mice treated with Ig-GAD2 were tested for cytokine production, we expected to see suppressive or Th2-associated cytokines, which usually drive bystander suppression. The results, however, showed that there was IL-10 production, but that this was accompanied by IFNγ (Fig. 5). The other surprise was that neutralization of IFNγ, but not IL-10, nullifies the therapeutic action of Ig-GAD2 and restores diabetes (Fig. 5). These findings provide support to prior observations showing that TCR transgenic IFNγ-producing GAD2-specific T cells prevent the onset of diabetes in an animal model of disease transfer (8). The question then is how can a well-defined inflammatory cytokine such as IFNγ mediate suppression of diabetes, which likely involves diverse T cell specificities? Given the recent observations indicating that IFNγ could interfere with differentiation of naive cells into Th17 (9, 10), and that IL-17, which is the product of Th17, displays pathogenic functions (39), we sought to test whether progression to diabetes involves the activity of Th17 and if so whether treatment with Ig-GAD2 affects these pathogenic T cells. Indeed, an increase of IL-17 was observed in NOD mice as they progressed toward diabetes (Fig. 6 A), and treatment with Ig-GAD2 reduced the frequency of IL-17–producing Th17 cells (Fig. 6 B). However, neutralization of IFNγ by anti-IFNγ antibody restored IL-17 production (Fig. 6 B). In support of this Ig-GAD2–induced IFNγ/IL-17 interplay is the observation that administration of rIL-17 with Ig-GAD2 nullified the therapeutic effect of Ig-GAD2. Also, neutralization of both IFNγ and IL-17 support protection, further justifying the IFNγ/IL-17 interplay. Moreover, polarized BDC2.5 Th17 cells transferred diabetes to NOD.scid mice, and neutralization of IL-17 inhibited such disease transfer (Fig. 6 F). Finally, IFNγ is mostly produced in the spleen, which provides a noninflammatory environment (Fig. 7, A and B) and likely acts to inhibit differentiation of naive cells into Th17 in this organ, leading to a diminished supply of pathogenic Th17 cells into the pancreas. In fact, upon treatment with Ig-GAD2, Th17 cells become undetectable in the spleen or pancreas, whereas IFNγ remained significant in the spleen to sustain a long-lasting inhibition of Th17 differentiation (Fig. 7, C and D). It is known that IFNγ signaling through IFNγ receptor (IFNγR), in conjunction with other inflammatory cytokines, interferes with β cell growth and induces apoptosis (40, 41). In the Ig-GAD2 treatment, the fact that IFNγ is produced in the spleen may play dual beneficial roles. It inhibits differentiation of pathogenic Th17 cells, allowing for clearance of infiltration and termination of islet inflammation and by being away from the islets its interference with β cell growth and death is prevented, hence proliferation of β cells. This also provides support to the dual pathogenic/protective role IFNγ plays in diabetes, which likely depends on the site of production and T cell differentiation (42). In fact, this goes well with the observation that neutralization of IL-17 did not protect against diabetes transferred by IFNγ-producing Th1 BDC2.5 cells, as these lymphocytes could home to the pancreas, where their IFNγ drives apoptosis of β cells (Fig. 6, E and F).

Overall, we suggest that adjuvant-free Ig-GAD2 induced the production of IFNγ in a noninflamed lymphoid organ, leading to inhibition of differentiation of naive cells into Th17 cells, culminating in diminished infiltration, formation of β cells and reversal of the diabetic process. The presence of IFNγ would inhibit differentiation of neighboring naive cells, thus suppressing diverse T cell specificities. For effective bystander suppression to occur, it may be that Th1 cells migrate to the PLN and inhibit differentiation of diverse T cells into Th17 cells. However, because Th1 cell were not detected in this organ, the likely alternative is that APCs loaded with β-cell antigens circulate from the pancreas to the spleen and subject diverse T cells to inhibition of differentiation by local IFNγ-producing GAD2-specific Th1 cells. Administration of exogenous IFNγ may protect against diabetes if targeted to the site of T cell differentiation during antigen stimulation, but away from the islets. It is also important to mention that the regimen is effective at late stages, but not before insulitis, possibly because availability of GAD2-specific T cells and production of IFNγ are delayed. In fact, Ig-INSβ was able to delay the disease when given at the preinsulitis stage (16), but was unable to counter the disease once the mice had progressed to the hyperglycemic stage (not depicted). Again, this supports the dynamics of different epitopes during disease initiation and progression.

Collectively, the findings suggest that this antigen-specific immunomodulation targets diverse pathogenic T cells to halt inflammation and drive an islet repair process that restores long-lasting normoglycemia.

MATERIALS AND METHODS

Mice

NOD (H-2^{g7}), NOD.BDC2.5, and NOD.scid mice were used according to the guidelines of the University of Missouri Columbia Animal Care and Use Committee.

Peptides

All peptides used in this study were purchased from Metabion and purified by HPLC to >90% purity. Glutamic acid decarboxylase 2 (GAD2) peptide corresponds to aa residues 206–220 (TYEIAPVFVLLEYVT) of GAD-65 (7). Hen egg lysozyme (HEL) peptide encompasses a nondiabetogenic epitope corresponding to aa residues 11–25 (AMKRHGLDNYRGYSL) of HEL (43). GAD2 and HEL peptides are presented to T cells in association with I-A^{g7} MHC class II molecules.

Ig chimeras

Ig-GAD2 and Ig-HEL express GAD2 and HEL peptide, respectively. This was accomplished by inserting the corresponding nucleotide sequence in place of the diversity segment within the complementarity determining region 3 (CDR3) of the heavy chain variable region of the 91A3 IgG2b, κ Ig (13–16). The fusion heavy chain gene was then transfected along with the parental κ light chain gene for expression as a complete self-Ig molecule, as previously described (11, 13–16). Large-scale cultures of transfectoma cells were performed in DME media containing 10% iron-enriched calf

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serum (BioWhittaker). Purification of the chimeras used separate columns of rat anti–mouse κ chain mAb coupled to CNBr-activated 4B Sepharose (GE Healthcare).

Islet cell purification

This was done according to a standard islet purification procedure (44). In brief, the pancreata were digested with collagenase type IV (Invitrogen), and islets were separated on a ficoll gradient (GE Healthcare).

T cell line and proliferation assay

A T cell clone specific for GAD2 peptide was generated in NOD mice as previously described (15). For presentation of Ig-GAD2, irradiated (3,000 rads) NOD female splenocytes (5×10^5 cells/50 μl/well) were incubated with graded amounts of either free peptide or Ig chimeras (100 μl/well), and 1 h later the GAD2-specific T cells (5×10^4 cells/well/50 μl) were added. Proliferation was measured by [^{3}H]thymidine incorporation assay.

Assessment of insulin autoantibody (IAA) seroconversion, hyperglycemia, and diabetes

Serum IAA was detected by ELISA using porcine insulin as antigen, as previously described (16). Assessment of blood glucose levels used test strips and an Accu-Chek Advantage monitoring system. A mouse is considered hyperglycemic or diabetic when the blood glucose level is 160–250 mg/dl or 300 mg/dl, respectively, for 2 consecutive weeks.

Ig-GAD2 treatment regimens

Treatment at the preinsulitis stage. Mice are given an i.p. injection of 300 μg Ig-GAD2 or Ig-HEL in 300 μl PBS at 4, 5, and 6 wk of age, a stage at which islet infiltration has begun and that is referred to as preinsulitis. The mice were monitored for blood glucose level up to 30 wk of age.

Treatment at the insulitis (IAA$^+$) stage. Mice are tested for IAA, and those who seroconvert between the ages of 8–11 wk are given a weekly i.p. injection of 300 μg of Ig-GAD2 or Ig-HEL in 300 μl PBS up to week 12. Subsequently, the mice received another 300 μg of Ig-chimera every 2 wk until the age of 24 wk. These mice were monitored for blood glucose level beginning at week 12 until 30 wk of age.

Treatment at the hyperglycemic stage. Mice began blood glucose level monitoring at 12 wk of age, and those who displayed a level of 160–250 mg/dl for 2 consecutive weeks between the ages of 14–30 wk were considered hyperglycemic. These mice were then subjected to a daily i.p. injection of 500 μg Ig-GAD2 or Ig-HEL for 5 d. Subsequently, the mice received another 500 μg of Ig-chimera every week for 15 or 25 consecutive weeks, and blood glucose levels were continuously monitored until 56 wk. These treatments are referred to as 15- and 25 wk-treatment regimen, respectively.

Histology

Pancreata were harvested from NOD females, fixed in 10% formalin, and embedded in paraffin. Sections of 8-μm thickness were cut 100 μm apart to prevent double counting the same islet. Four sections per pancreas were stained with hematoxylin and eosin and analyzed by light microscopy. Insulitis scoring was performed according to the following criteria: severe insulitis, 50% or higher of the islet area is infiltrated; mild insulitis, <50% of the islet area is infiltrated; periinsulitis, infiltration is restricted to the periphery of islets; and no insulitis, absence of cell infiltration.

Immunohistochemistry

Evaluation of cell division by insulin-producing β cells was done as follows: Ig chimera–treated mice were injected i.p. with 100 mg/kg of BrdU in PBS (Sigma-Aldrich), 3 h before euthanasia. Pancreata and intestine were harvested and fixed, and sections were prepared as described in the previous section. For assessment of insulin production, the sections were stained with primary guinea pig anti-insulin antibodies, incubated with biotinylated goat anti–guinea pig antibodies, and visualized by saturation with Streptavidin-alkaline phosphatase using the chromagen, 5-Bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium. For detection of BrdU incorporation, the sections were counter stained with biotinylated anti-BrdU antibody (Zymed), treated with Streptavidin-horseradish peroxidase, and visualized with the chromagen 3-amino-9-ethylcarbazole.

Cytokine assays

Splenocytes (5×10^5 cells/well) were incubated with 30 μg/ml of free peptide or 5 μg/ml anti-CD3 antibody for 48 h, and cytokines in the supernatant were measured by ELISA and ELISPOT, as previously described (45).

Flow cytometry

For staining of CD4, CD25, and CD62L, cells were harvested from spleens and pancreatic lymph nodes and incubated with anti-CD4-PE, biotin-conjugated anti-CD25 (or isotype control biotin-conjugated rIgM), and anti-CD62L-FITC (or isotype control rIgG2a-FITC) for 30 min at 4°C. Subsequently, the cells were washed and stained with PerCP-conjugated streptavidin for 30 min at 4°C. The cells were washed, fixed with 4% formaldehyde for 20 min at room temperature, and then analyzed. All antibodies were purchased from BD PharMingen.

For intracellular Foxp3 staining, cells from spleens and pancreatic lymph nodes were first stained with anti-CD4-PE and biotin-conjugated anti-CD25 antibodies. This was followed by PerCP-conjugated streptavidin staining. The cells were fixed with Fix/Perm buffer (eBioscience), washed with permeabilization buffer (eBioscience), and stained with anti-Foxp3-FITC antibody (clone FJK-16s; eBioscience), or isotype control rIgG2a-FITC.

For intracellular cytokine analysis of IL-10 and IFNγ, the splenic cells (2×10^6 cell/ml) were stimulated with free peptide (30 μg/ml) for 6 h followed by 10 h incubation with brefeldin A (10 μg/ml) to block cytokine secretion and facilitate intracellular accumulation. The antibodies used were PerCP-anti-CD4 (RM4-5), biotin-anti-Vβ8.1/8.2, PE-anti-IFNγ (XMG1.2), and FITC-anti-IL-10 (JESS-16E3; all from BD Biosciences). Isotype-matched controls were included in all experiments. Events were collected on a FACScan flow cytometer and analyzed with CellQuest software (Becton Dickinson).

T cell polarization

Naive splenocytes were isolated from 4-wk-old NOD.BDC2.5 transgenic mice and activated with soluble anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) antibodies for 3 d in 10% FCS-DME media under Th17 polarizing (TGFβ [3 ng/ml], IL-6 [20 ng/ml], anti-IFNγ antibody [10 μg/ml], and anti–IL-4 antibody [10 μg/ml]) and nonpolarizing conditions. Supernatant from activated cells was tested for IFNγ and IL-17 by ELISA, and the cells were used for adoptive transfers.

Adoptive transfer experiments

For disease transfer by Th17, 10×10^6 naive, nonpolarized and Th17-polarized cells were injected i.v into NOD.scid (4–6-wk-old) mice. Additional groups of mice received IL-17–neutralizing antibody along with the T cell transfer to serve as controls. Anti-IL-17 antibody (TC11-18H10; 200 μg/mouse) was given on the day of transfer, and 2 additional injections were given at day 4 and 16 after transfer.

Statistical analysis

The χ^2 test was used for incidence of diabetes analysis among experimental and control groups. For the rest of the experiments, P values were calculated with the two-tailed Student's unpaired *t* test.

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EXHIBIT R

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TITLE:	Methods and compositions for preventing the onset of type 1 diabetes (*as amended*)		

DECLARATION UNDER 37 C.F.R. § 1.132

I, Habib Zaghouani, do hereby declare and say:

1. I am a citizen of the United States and my current residential address is 1608 Brookfield Manor, Columbia, Missouri, 65203.

2. I obtained my undergraduate degree in biochemistry from University of Paris, France in 1981. I obtained a Ph.D. in immunology from the University of Paris/Cancer Research Institute, France in 1987.

3. I am presently the J. Lavenia Edwards Chair in Pediatrics, Director, Center for Cellular and Molecular Immunology and Professor, Department of Molecular Microbiology & Immunology and Department of Child Health at the University of Missouri.

4. I have over one hundred publications and abstracts in the field of immunology. Please refer to the copy of my *curriculum vitae* in attached Appendix A for more details.

5. I am a named inventor on the '070 application as well as on related co-pending application serial numbers: 10/681,788; 11/510,411; and 11/425,084.

6. I have performed experiments examining the impact of administration, initiated at the pre-diabetic stage, of soluble Ig-GAD2 to NOD mice over a period of 56 weeks. Data are provided in attached Appendix B.

7. NOD mice were assessed for blood glucose beginning at week 12 of age. Those mice

that reached glucose levels of 160 – 250 mg/dl between week 14 to 25 received the following Ig-GAD2 regimen: 500 μg of soluble Ig-GAD2 i.p. daily for 5 days and then weekly injections thereafter for either 15 or 25 weeks. Blood glucose monitoring was performed during this period.

8. Overall, 100% of mice that became pre-diabetic at the age of 14 – 25 weeks and that were not treated with Ig-GAD2 progressed to diabetes (blood sugar level ≥300 mg/dl glucose) within 5 weeks after diagnosis of the pre-diabetic stage. Moreover, 60% of mice undergoing the 15-week treatment regimen were protected against diabetes throughout the 25 week post-hyperglycemia monitoring period. Interestingly, one mouse (Figure 1 B, left panel, open stars) progressed to diabetes by 5 weeks of treatment and 3 mice (Figure 1 B, plus, open diamond, and open pentagon) had similar disease manifestations shortly after interruption of the treatment.

9. When the regimen was extended to 25 weeks, 100% of the Ig-GAD2 treated animals were protected (Figure 1 A, right panel) and normoglycemia was restored in all mice (Figure 1 B, right panel). This status persisted throughout the duration of the study, which was terminated when the mice were 52 to 56 weeks of age.

10. Detailed histopathologic analysis from the mice was performed. While most of the islets in hyperglycemic and diabetic control mice exhibited intrainsulitis (Figure 2, panels 1, 2 and 3), the majority of islets in treated mice were not inflamed (Figure 2, panel 4) or had only mild periinsulitis (Figure 2, panels 5 and 6).

11. Overall, the histopathologic analysis indicated that treated mice had significantly greater number of islets when compared to both hyperglycemic and diabetic mice (Figure 3). The number of insulin-positive islets also increased from 14 per pancreas at the prediabetic stage to 29 per pancreas upon treatment with soluble Ig-GAD2. Analysis of islet infiltration scores among the different groups of mice indicated that the 15-week treatment group had a higher number of islets with periinsulitis (38% vs. 30%) or no insulitis (35% vs. 17%) relative to the hyperglycemic stage (Figure 4). On the other hand, the number of islets with severe- and mild-intrainsulitis were reduced in the treated versus hyperglycemic mice (8% and 19% vs. 22% and 31%, respectively) (Figure 4).

12. Surprisingly, in the 25-week treatment group, although the total number of islets was reduced to that of the hyperglycemic stage, most of these islets exhibited no (60%), peri- (28%) or mild intra- (12%) insulitis (Figure 4). Overall, the treatment with Ig-GAD2 led to a significant increase in the number of noninflammed ("healthy") islets that restored and maintained normoglycemia.

13. An experiment was performed to determine whether the healthy islets discussed above were a result of a regression of inflammation and/or regeneration of beta cells. To address this question, the treated mice were injected with the proliferation indicator 5-bromo-2'-deoxyuridine (BrdU), sacrificed and pancreatic sections were stained with anti-insulin and anti-BrdU antibodies and analyzed for BrdU incorporation and insulin production.

14. BrdU staining was visible in the highly proliferative luminal intestinal cells but these had no staining with anti-insulin antibody (Figure 5). Islets of non-diabetic 5-week old NOD mice were positive when stained with anti-insulin antibody, but did not incorporate BrdU, suggesting that these insulin-producing beta cells were not newly generated cells (Figure 6). Thus, under normal circumstances insulin production emanates from existing beta cells whose nuclei do not incorporate BrdU giving a minimal number of BrdU/insulin double-positive ($BrdU^+/insulin^+$) beta cells (Figure 7). Sections from hyperglycemic mice showed very few insulin-producing beta cells and no BrdU incorporation (Figure 8) resulting in an insignificant number of $BrdU^+/insulin^+$ beta cells (Figure 7). In contrast, islets from the 25-week treatment group showed beta cells that stained positive for insulin and were either BrdU negative (previously generated beta cells) or BrdU positive (newly generated beta cells) (Figure 9). Notably, the number of these insulin-producing regenerating beta cells was significantly increased in all five mice in which treatment restored normoglycemia (Figure 7).

15. Interestingly the total number of regenerating cells producing insulin ($Insulin^+/BrdU^+$) was low and may not solely account for the restoration of normoglycemia. Insulin-positive / BrdU-negative islet cells may have also contributed to the control of blood glucose level and these likely represent a combination of newly regenerated and

previously existing beta cells. There were also numerous BrdU positive / insulin-negative islet cells that likely represent newly regenerating cells that are not yet producing abundant insulin (Figure 9).

16. Splenic cells from recovered mice were then stimulated with GAD2 peptide and assessed for both suppressive and inflammatory cytokines. The results indicated that although no measurable IL-4 or TGFβ was detected (not depicted), there was significant IFNγ and IL-10 production by these cells relative to the control HEL peptide (Figure 10).

17. When *in vivo* cytokine neutralization was performed along with soluble Ig-GAD2 treatment, the recovery persisted with anti-IL 10 treatment but was nullified by removal of IFNγ (Figure 11). These results indicate that IFNγ (but not IL-10), contrary to IFNγ's well-defined inflammatory function, is likely involved in modulation of inflammation and restoration of normoglycemia.

18. Th17 cells represent a newly defined subset of pathogenic T cells whose development can be facilitated by TGFβ and IL-6 or interfered with by IFNγ or IL-27. Because soluble Ig-GAD2 treatment induces IFNγ, we sought to determine whether restoration of normoglycemia involves interference with IL-17 production. As such, an experiment was performed to assess whether IL-17 is produced by NOD T cells and, if so, to follow its pattern of secretion during disease progression.

19. IL-17 was evident upon IAA-seroconversion and increased significantly when the mice progressed to hyperglycemia and diabetes. Treatment with soluble Ig-GAD2 at the hyperglycemic stage significantly reduced the frequency of GAD2-specific IL-17 producing cells as measured by spot formation (data not shown). However, neutralization of IFNγ by administration of anti-IFNγ antibody along with Ig-GAD2 restored even higher frequency of Th17 cells which was likely caused by complete neutralization of IFNγ (data not shown). It is therefore likely that the restoration of diabetes by neutralization of IFNγ during treatment with Ig-GAD2 as seen in Figure 11 is actually caused by restoration of Th17.

20. At the time of filing of the '070 application T1D was suspected to involve multiple

autoantigens and the initiating antigen was unknown. As such, a person of ordinary skill in the art reading the literature at that time would have expected that induction of tolerance, if even possible, would require modulation of diverse T cell specificities through a combination of peripheral tolerance and bystander suppression.

21. It was known prior to filing of the '070 application that cross-linking of Fc receptors on target cells by antigen-antibody complexes could trigger the production of cytokines such as IL-10 which are important for down regulating T cells engaged to antigen presenting cells as well as neighboring T cells (*e.g.* bystander suppression).

22. Additionally, at the time of the '070 application, aggregation of Igs was known to confer effector functions associated with the Fc fragment without the need for complex formation.

23. In view of the foregoing, at the time the '070 application was filed, a person of ordinary skill in the art would not have believed that a *soluble* Ig-peptide chimera would be effective at treating T1D because such a person would not have expected a soluble Ig chimera to cross-link Fc receptors and stimulated cytokines (*e.g.* IL-10) by APCs so as to stimulate bystander suppression.

24. Surprisingly, we have found that soluble Ig-GAD2, despite the fact that it does not induce cross-linking of Fc receptors, delayed T1D when administered at the preinsulitis stage and reversed diabetes when given at the prediabetic stage whereas aggregated Ig-GAD2 (which would be expected to cross-link Fc receptors and induce IL-10 secretion by APCs) did not delay diabetes when given after IAA seroconversion (unpublished data).

25. In my opinion, at the time of filing of the present application a person of ordinary skill in the art would not have expected that the presently claimed soluble Ig-GAD2 construct but not the aggregated Ig-GAD2 construct would delay T1D when administered after IAA seroconversion—a relatively advanced stage of disease.

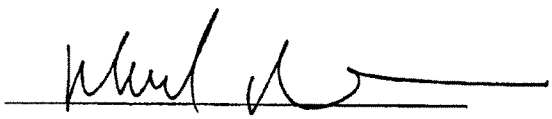
26. In my opinion, a person of ordinary skill in the art, at the time of filing the present application, would not have expected that an antigen-specific single-epitope therapy such as soluble Ig-GAD2 would restore normoglycemia in hyperglycemic NOD mice.

27. It would have been particularly surprising to the person of ordinary skill in that art that soluble Ig-GAD2 was able to restore normoglycemia through an IL-10-independent mechanism. Such a person would certainly not have predicted that soluble Ig-GAD2 would delay T1D and reverse disease in pre-diabetic mice by inducing IFNγ to suppress IL-17 which is contrary to IFNγ's well known inflammatory function.

28. Moreover, in my opinion, at the time of filing the present application a person of ordinary skill in the art would not have expected that treatment with the claimed soluble Ig-GAD2 construct would lead to an increase in the number of healthy, insulin positive pancreatic islet cells in NOD mice by comparison with controls.

29. I declare that all statements made herein of my own knowledge and are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

July 15, 2009
Date



Habib Zaghouani, PhD

APPENDIX A

Curriculum Vitae Habib Zaghouani

EDUCATION

Ph.D.	1987	Immunology, University of Paris/Cancer Research Institute, Paris, France.
M.S.	1983	Immunology, University of Paris/Pasteur Institute, Paris, France.
B.S.	1981	Biochemistry, University of Paris, Paris, France.

POSITIONS AND RESEARCH EXPERIENCE

2006-present Director, Center for Cellular and Molecular Immunology, The University of Missouri School of Medicine, Columbia, MO

2006-present: J. Lavenia Edwards Chair in Pediatrics, the University of Missouri School of Medicine, Columbia, MO

2006-present: Professor, Department of Child Health, the University of Missouri School of Medicine, Columbia, MO.

2001-present: Professor, Department of Molecular Microbiology and Immunology, the University of Missouri School of Medicine, Columbia, MO.

2000-2001: Associate Professor, Department of Microbiology, the University of Tennessee, Knoxville, Tennessee.

1994-2000: Assistant Professor, Department of Microbiology, the University of Tennessee, Knoxville, Tennessee.

1990-1994: Research Assistant Professor, Department of Microbiology, Mount Sinai School of Medicine, New York.

1987-1989: Postdoctoral Fellow, Department of Microbiology, Mount Sinai School of Medicine, New York. Mentor: Dr. Constantin A. Bona.

1983-1987: Graduate Research Assistant, Ph.D. candidate, Immunology, University of Paris/Cancer Research Institute, Paris, France. Mentor: Dr. Marc Stanislawski.

1981-1983: Graduate Research Assistant, M.S. candidate, Immunology, Pasteur Institute, Paris, France. Director: Dr. Arthur Dony Strosberg.

RESEARCH GRANT SUPPORT

A. Principal Investigator

Active

1). 2RO1 NS 037406, National Institutes of Health, March 2004 - February 2009. Modulation of autoreactive T cells. PI: Habib Zaghouani.

2). 1RO1 DK 065748, National Institutes of Health, April 2005-March 2008. Immune tolerance against type I diabetes in mice. PI: Habib Zaghouani.

3). 2RO1 AI 48541, National Institutes of Health, May 2006- April 2011. Regulation of neonatal immunity. PI: Habib Zaghouani.

4). 1R21 AI 068746, National Institutes of Health. July 2007 – June 2009. Mimotopes against type I diabetes. PI: Habib Zaghouani.

Pending

1). 1RO1 NS057194-A2, National Institutes of Health, April 2008 - March 2013. Regulation of autoimmune encephalomylitis. PI: Habib Zaghouani. **8.7 percentile**
(a)

2). 2RO1 DK 065748-01, National Institutes of Health, April 2008-March 2013. Immune tolerance against type I diabetes in mice. PI: Habib Zaghouani. **35 percentile**

B. Co-Investigator, Mentor, or Key Personnel

Active

T32 GM008396, National Institute of General Medical Sciences (NIGMS), July 1991-June 2012. Molecular Basis of Gene Expression and Signal Processing. PI: Mark Hannink (Zaghouani: Mentor).

T32 RR007004, National Institutes of Health, July 2005-June 2010, Postdoctoral Training in Comparative Medicine. PI: Craig Franklin (Zaghouani: Mentor).

T90 DK71510, National Institutes of Health, September 2004 – August 2009. Bench and Back: Clinical biodetectives training. PI: Mark Milanick (Zaghouani: Mentor).

R90 DK71510, National Institutes of Health, September 2004 – August 2009. Bench and Back: Clinical biodetectives training. PI: Mark Milanick (Zaghouani: Mentor).

KO8 AR048671, National Institutes of Health, June 2005-April 2008, Cytokine regulation of collagen-induced arthritis. PI: Robert Ortman (Zaghouani: Mentor).

1G20 RR021327, National Institutes of Health, September 2004-August 2009. Equipment for the MU Life Sciences Center. PI: Lon Dixon, (Zaghouani: Key personnel).

1 G20 RR019711, National Institutes of Health, September 2004-Agust 2009. Renovation of MU Medical School Vivarium. PI: Lon Dixon. (Zaghouani: Key personnel).

U19AT003264-01, National Institutes of Health, September 2005 – August 2009. TICIPS: HIV/AIDS, Secondary Infections and Immune Modulation. Center grant. PI: William Folk (Zaghouani: Faculty Member).

Research Foundation Grant, Arthritis Foundation, April 2006 – May 2008. Synoviolin is a target for arthritis. PI: Deyu Fang (Zaghouani: Mentor).

C. Previous Support (PI: Zaghouani, H)

1). R21 AI 062796, National Institutes of Health, July 2005-June 2007. Immune tolerance in the newborn mouse. Yearly direct cost $150,000. PI: Habib Zaghouani. No cost extension 11/30/2007

2). 1RO1 AI48541, National Institutes of Health, May 2001- April 2006. Regulation of neonatal immunity. Yearly direct cost: $175,000. PI: Habib Zaghouani.

3). Astral Inc, October 2001- September 2004. Development of Approaches to Combat Autoimmunity. PI: Habib Zaghouani.

4). RO1NS37406, National Institutes of Health, January 2000- December 2004. Modulation of autoreactive T cells. PI: Habib Zaghouani

5). RG2967B-3, National Multiple Sclerosis Society, October 2002 – March 31, 2004 Down-regulation of encephalitogenic T cells. PI: Habib Zaghouani.

6). RG2967A2/1, National Multiple Sclerosis Society, April 99 - March 2002. Down-regulation of encephalitogenic T cells. PI: Habib Zaghouani.

7). Astral Inc: March 95 - July 2001. A novel approach to delete encephalitogenic T cells. PI: Habib Zaghouani.

8). RG2778A1/1, National Multiple Sclerosis, April 96 - March 1999. A deletional strategy for encephalitogenic T cells. PI: Habib Zaghouani.

9). Astral Inc:, September 97- August 99. Generation of human Ig chimeras carrying wild type or antagonist forms of myelin peptides. PI: Habib Zaghouani.

10). 1R41AI47496, (STTR): National Institutes of Health, September 2000-August 2001. Treatment of EAE using a novel delivery system. . Co-PI: Habib Zaghouani.

TEACHING EXPERIENCE

2004:	Microbiology 205 (Medical Microbiology) 3 credit hours, 8 lecture contact hours, 170 student, Spring semester, University of Missouri School of Medicine, Columbia.
2002-present:	Microbiology 304 (Immunology) 3 credit hours, 14 lecture contact hours, 30 students, Fall semester, Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia.
2002-present	Microbiology 407 (advanced Immunology) 4 credit hours, 9 lecture contact hours, 18 students, Spring semester, Molecular Microbiology and Immunology, University of Missouri School of Medicine, Columbia.
2001-present:	Bio 4952, Undergraduate research, 3 credit hours, 1-2 students, Fall and Winter semesters
2001-present:	Bio 4950, Undergraduate research, 3 credit hours, 2-3 Students, Fall and Winter semesters
2001-present:	Direct Immunology Journal Club, 1hour/week all year around, 40 student, postdocs and faculty members
1995-2001:	Microbiology 430 (Immunology), 3 credit hours, 45 lecture contact hours, 100-120 students, Fall semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Co-direct Microbiology 602 (Microbial Pathogenesis Journal Club), 1 credit hour, 15 lecture contact hours, 10-15 students, Fall semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Co-direct Microbiology 603 (Immunology Journal Club), 1 credit hours, 15 lecture contact hours, 10-15 students, Spring semester, Microbiology, The University of Tennessee, Knoxville.
1995-2001:	Microbiology 401 (Undergraduate Research), 3 credit hours, 1-2 students per semester, Microbiology, The University of Tennessee, Knoxville.

1998: Microbiology 630 (Topics in Immunology), 3 credit hours, 10 lecture contact hours, 20 students, Spring semester, (Seminar Series) Microbiology, The University of Tennessee, Knoxville.

1998-2001: Microbiology 493 (Independent Study in Immunology), 6 students, 10 lecture contact hours, spring, Microbiology, The University of Tennessee, Knoxville.

1992-1994: 600-level Immunology course, 3 credit hours, 6 lecture contact hours, 10 students, spring, Microbiology, Mount Sinai School of Medicine, New York.

HONORS AND AWARDS

2006. Speaker, Keystone Symposia on Tolerance Autoimmunity and Immune Regulation. March 21-26, 2006. Beaver Run Resort, Breckenridge, Colorado. Presentation title: Tregs for or against diabetes.

2004: Research Equipment Award for the purchase of an ELISPOT Analyzer, Office of Research, The University of Missouri,

2003: Keystone Symposia Scholarship ($1,000) for poster presentation by Hyun-Hee Lee, a graduate student in the laboratory, the meeting was held in Snowbird, UT

2003: Honorable citation for poster presentation by Randal Gregg, a graduate student in the laboratory. Life Science week, University of Missouri-Columbia.

2001: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

2000: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

2000: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of research Administration, The University of Tennessee, Knoxville.

1999: Chancellor's nomination for Howard Hughes Medical Institute Assistant Investigator Appointment, The University of Tennessee, Knoxville.

1999: Biological Equipment Award, Office of Research Administration/Science Alliance/Genome Science and Technology/Division of Biology, The University of Tennessee, Knoxville.

1999: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

1999: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of research Administration, The University of Tennessee, Knoxville.

1998: Science Alliance Research Excellence Award, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.

1998: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of Research Administration, The University of Tennessee, Knoxville.

1997: Biological Equipment Award, Office of Research Administration/Science Alliance/ Division of Biology/ Department of Microbiology, The University of Tennessee, Knoxville.

1997: Exhibit, Performance, and Publication Expense Award, Faculty Senate Research Council and Office of Research Administration, The University of Tennessee, Knoxville.

1990: Research Excellence Award, Alliance Pharmaceutical Corporation. San Diego, CA.

1987-1988: Scientist Exchange Award (Postdoctoral Fellowship), French Cancer Society, Paris, France.

1984-1987: Graduate Student Scholarship, French Cancer Society, Paris, France.

PROFESSIONAL SERVICE

2007: Chair, Block symposium, regulation of immune cell development and function, American Association of Immunologis, Miami, FL.

2006-2010: Panel member, Hypersensitivity, Autoimmune and Immune-mediated Diseases (HAI) study section.

2006: Chair, Block symposium, treatment of autoimmune disease, American Association of Immunologis, Boston, MA.

2006: Review panel member, research proposals on Neurosciences, La Marato de TV3 Foundation, Catalan Agency For Health Technology Assessment And Research

2005: Chair, Block symposium, Cytokines and autoimmunity, American Association of Immunologis, Experimental Biology Meeting, San Diego, CA.

2004: Panel member, NIAID Biodefence Workshop, Immunization and Vacination in Special Populations, Division of Allergy, Immunology and transplantation, NIH, Bethesda, MD

2004:	Chair, Block symposium, Tolerance and regulation of autoimmunity, American Association of Immunologis, Experimental Biology Meeting, Washington DC.
2004-present:	Adhoc Reviewer, TTT Study section, National Institutes of Health
2004-present:	Adhoc Reviewer, HAI Study section, National Institutes of Health
2003	Adhoc Reviewer, IMS Study Section, National Institutes of Health
2003	Adhoc Reviewer, ALY Study Section, National Institutes of Health
2003-present:	Member, Molecular Biology Program, University of Missouri-Columbia
2003-present:	Member, Genetics Area Program, University of Missouri-Columbia
2003-present:	Member, Veterinary Pathobiology Area Program, University of Missouri-Columbia
2003-present	Scientific Consultant, Division of endocrinology and Diabetes, Unversity of Missouri, Kansas City, MO
2002-2004:	Scientific Consultant, Alliance Pharmaceutical, San Diego, CA.
2001-present:	Member of The Graduate Student Recruitment Committee, Department of Molecular Microbiology and Immunology, The University of Missouri School of Medicine, Columbia.
2000-2001:	Adhoc Reviewer, BM-1 Study Section, National Institutes of Health
1992-2000:	Editorial board member: *Viral Immunology*
1989-present:	Reviewer: Immunology Journals
2000:	Guest Editor, International Review of Immunology
2000-2001:	Chair, Graduate Student Advisory Committee, Genome, Science, and Technology program, Oak Ridge National Laboratories and The University of Tennessee, Knoxville.
1995-2001:	Member of The Graduate Student Recruitment Committee, Department of Microbiology, The University of Tennessee, Knoxville.
1998:	Member of Faculty Search Committee, Department of Comparative Medicine, College of Veterinary Medicine, The University of Tennesee, Knoxville.
1999:	Panel Member: NIH/NCI, Small Business Innovation Research (SBIR)/Small Business Technology Transfer (STTR) Grant program.

Flexible system to advance innovative research for cancer drug discovery by small business panel.

PROFESSIONAL MEMBERSHIP

2006-present: Member of the Henry Kunkel Society
1998-present: Member of the Society for Neuroscience
1992-present: Member of the American Association for the Advancement of Science.
1992-present: Member of the American Association of Immunologists.

PUBLICATIONS

Manuscripts published in peer-review journals

1. Bot, A., D. Smith, B. Phillips, S. Bot, C. Bona, and H. Zaghouani. (2006). Immunologic control of tumors by *in vivo* FcgR-targeted antigen loading in conjunction with dsRNA-mediated immune modulation. *J. Immunol.* 176:1363-1374.

2. 58. Caprio-Young, J., J. J. Bell, H-H. Lee, J. S. Ellis, D. M. Nast, G. Sayler, B. Min, and H. Zaghouani. (2006). Neonatally Primed Lymph Node but not Splenic T Cells Display a Gly- Gly Motif Within the T Cell Receptor Beta Chain Complementarity Determining Region 3 (CDR3) That Controls Affinity and Lymphoid Organ Retention. *J. Immunol.* 176:357-364.

3. Yu, P., R. K. Gregg, J. J. Bell, J. S. Ellis, R. Divekar, H-H Lee, R. Jain, H. Waldner, J. C. Hardaway, M. Collins, V. K. Kuchroo, and H. Zaghouani. (2005). Specific T regulatory cells (Tregs) display broad suppressive functions against experimental allergic encephalomyelitis upon activation with cognate antigen. *J. Immunol.* 174:6772-6780.

4. Gregg, R. K., J. J. Bell, H-H. Lee, R. Jain, S. J. Schoenleber, R. Divekar, and H. Zaghouani (2005). IL-10 diminishes CTLA-4 expression on islet-resident T cells and sustains their activation rather than tolerance. *J. Immunol.* 174: 662-670.

5. Gregg, R. K., R. Jain, S. J. Schoenleber, R. Divekar, J. J. Bell, H-H. Lee, P. Yu, and H. Zaghouani. (2004). A sudden decline in active membrane-bound TGFβ impairs both T regulatory cell function and protection against autoimmune diabetes . *J. Immunol.* 173:7308-7316.

6. Li, L, H-H. Lee, J. J. Bell, R. K. Gregg, J. S. Ellis, A. Gessner, and H. Zaghouani. (2004). IL-4 Utilizes an Alternative Receptor to Drive Apoptosis of Th1 Cells and Skews Neonatal Immunity Towards Th2. *Immunity.* 20: 429-440.

7. Bell, J. J., B. Min, R. Gregg, H-H. Lee, and H. Zaghouani. (2003). Break of neonatal Th1 tolerance and exacerbation of experimental allergic encephalomyelitis by interference with B7 costimulation. *J. Immunol.* 171:1801-1808.

8. Legge, K. L., Gregg, R. K. Maldonado-Lopez, R., Li, L., Caprio, J. C., Moser, M., and Zaghouani, H. (2002). On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. J. Exp. Med. 196:217-227.

9. Pack, C. D., Cestra, A. E., Min, B., Legge, K. L., Li, L., Caprio, J. C., Bell, J. J., Gregg, R. K., and Zaghouani, H. (2001). Neonatal exposure to antigen primes the immune system to develop responses in various lymphoid organs and promotes bystander regulation of diverse T cell specificities. *J. Immunol.* 167:4187-4195.

10. Li, L., Legge, K. L, Min, B., Bell, J. J, Gregg, R., Caprio, J. and Zaghouani, H. (2001). Neonatal immunity develops in a transgenic TCR transfer model and reveals a requirement for elevated cell input to achieve organ-specific responses. *J. Immunol.* 167:2585-2594

11. Min, B., Legge, K. L., Li, L., Caprio, J. C., Gregg, R. K., Bell, J. J., and Zaghouani, H. (2001). Defective expression of CD40L undermines both IL-12 production by antigen presenting cells and up-regulation of IL-2 receptor on splenic T cells and perpetuates INFγ-dependent T cell anergy. *J. Immunol.* 166:5594-5603.

12. Day, R. B., Okada, M., Ito, Y., Tsukada, K., Zaghouani, H., Shibuya, N., and Stacey, G. (2001). Binding site of chitin oligosaccharides in the soybean plasma membrane. *Plant. Phys.* 126:1-12.

13. Legge, K. L., Min, B., Caprio, J. C., Li, L., Gregg, R. K., Bell, J. J., and Zaghouani, H. (2000). Coupling of peripheral tolerance to endogenous IL-10 promotes effective modulation of myelin-activated T cells and ameliorates experimental allergic encephalomyelitis. *J.Exp.Med.* 191:2039-51.

14. Anderson, A. C., Nicholson, L. B., Legge, K. L., Turchin, V., Zaghouani, H., and Kuchroo, V. K. (2000). High frequency of auto-reactive myelin proteolipid protein (PLP)-specific T cells in the periphery of naïve mice: mechanisms of selection of the self-reactive repertiore. *J. Exp. Med.* 191:761-770.

15. Min, B., Legge, K. L., Caprio, J. C., Li, L., Gregg, R., and Zaghouani, H. (2000). Differential control of neonatal tolerance by antigen dose versus extended exposure and adjuvant. *Cell. Immunol.* 200 :45-55.

16. Legge, K. L., Min, B., Pack, C. D., Caprio, J. C., and Zaghouani, H. (1999). Differential presentation of an altered peptide within fetal central and peripheral organs supports an avidity model for thymic T cell development and implies a peripheral re-adjustment for activation. *J. Immunol.* 162:5738-46.

17. Min, B., Legge, K. L., Pack, C. D. and Zaghouani, H. (1998). Neonatal exposure to a self peptide-Ig chimera circumvents the use of adjuvant and confers resistance to autoimmune disease by a novel mechanism involving IL-4 lymph node deviation and INFγ-mediated splenic anergy. *J. Exp. Med.* 188:2007-17.

18. Legge, K. L., Min, B., Cestra, A.E., Pack, C. D., and Zaghouani, H. (1998). T cell receptor agonist and antagonist exert in vivo cross-regulation when presented on Immunoglobulins. J. Immunol. 161:106-11.

19. Legge, K. L., Min, B., Potter, N.T., and Zaghouani, H. (1997). Presentation of a T cell receptor antagonist peptide by immunoglobulins ablates activation of T cells by a synthetic peptide or protein requiring endocytic processing. *J. Exp. Med.* 185:1043-53.

20. Brumeanu, T-D, Dehazya, P., Wolf, I., Bot, A., Bona, C., and Zaghouani, H. (1996). Engineering of double antigenized Igs carrying B and T cell epitopes. Immunotechnology 2:85-95.

21. Brumeanu, T-D., Zaghouani, H., and Bona, C. (1995). Purification of antigenized immunoglobulins derivatized with monomethoxypolyethylene glycol. *J. Chromatogr.* 696:219-25.

22. Brumeanu, T-D., Zaghouani, H., Elahi, I., Daian, C. and Bona, C. (1995). Derivatization with monomethoxypolyethylene glycol of Igs expressing viral epitopes obviates adjuvant requirement. *J. Immunol.* 154:3088-95.

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EXHIBIT S

APPENDIX B

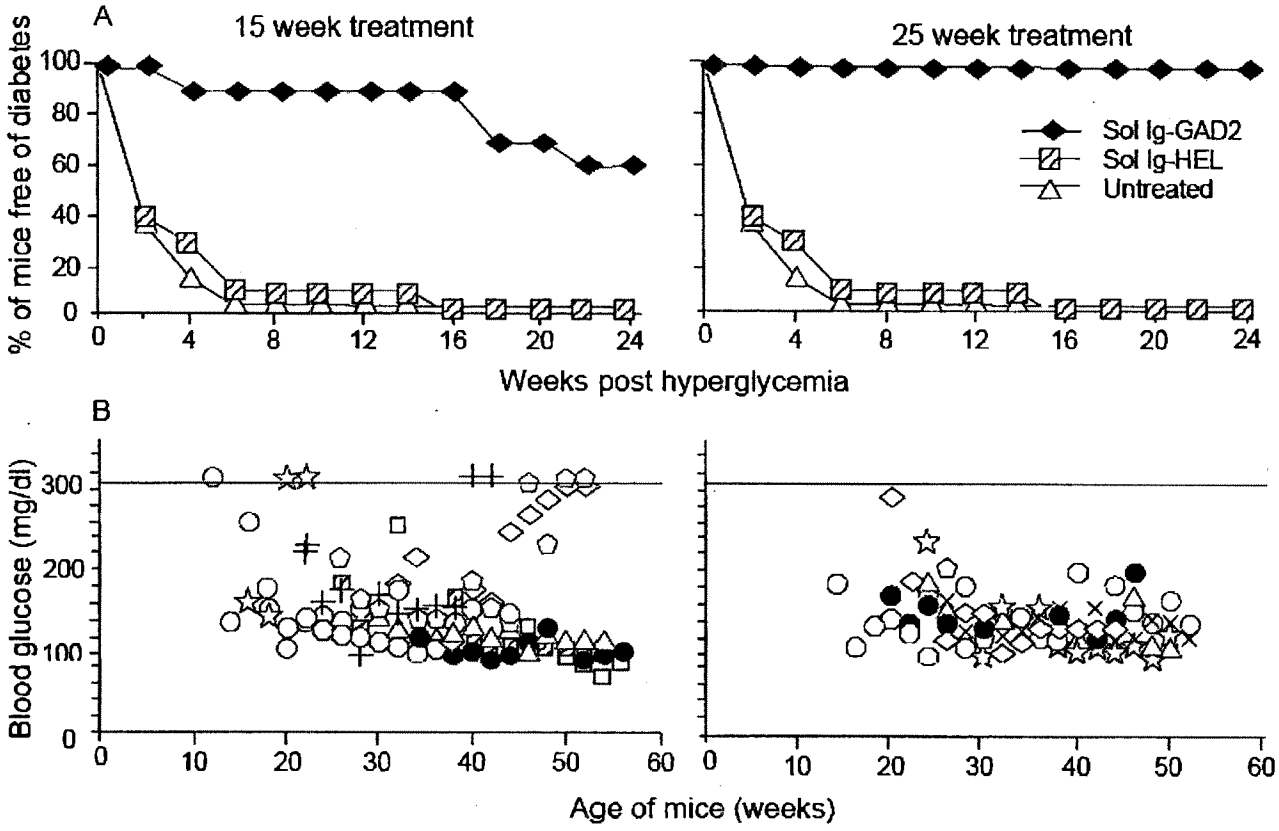


Figure 1

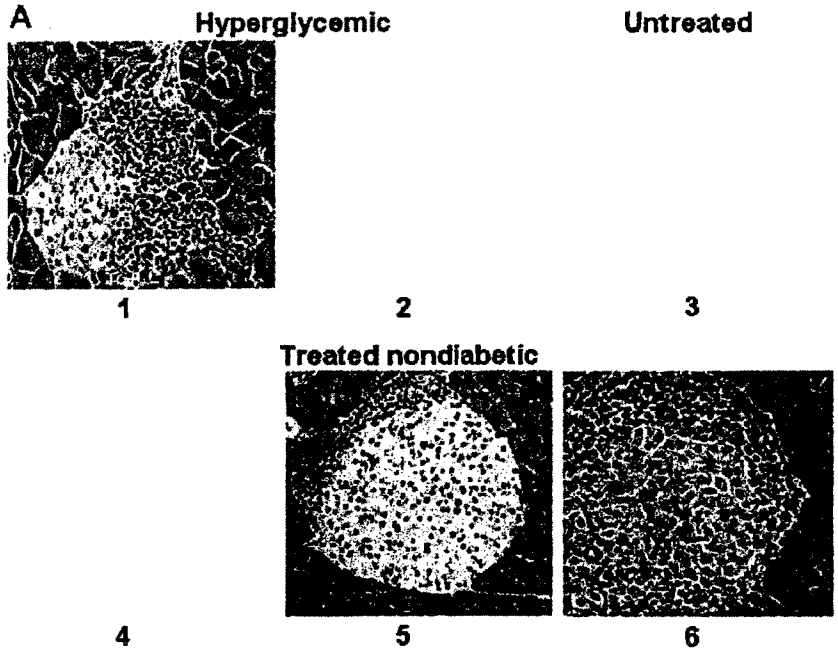


Figure 2

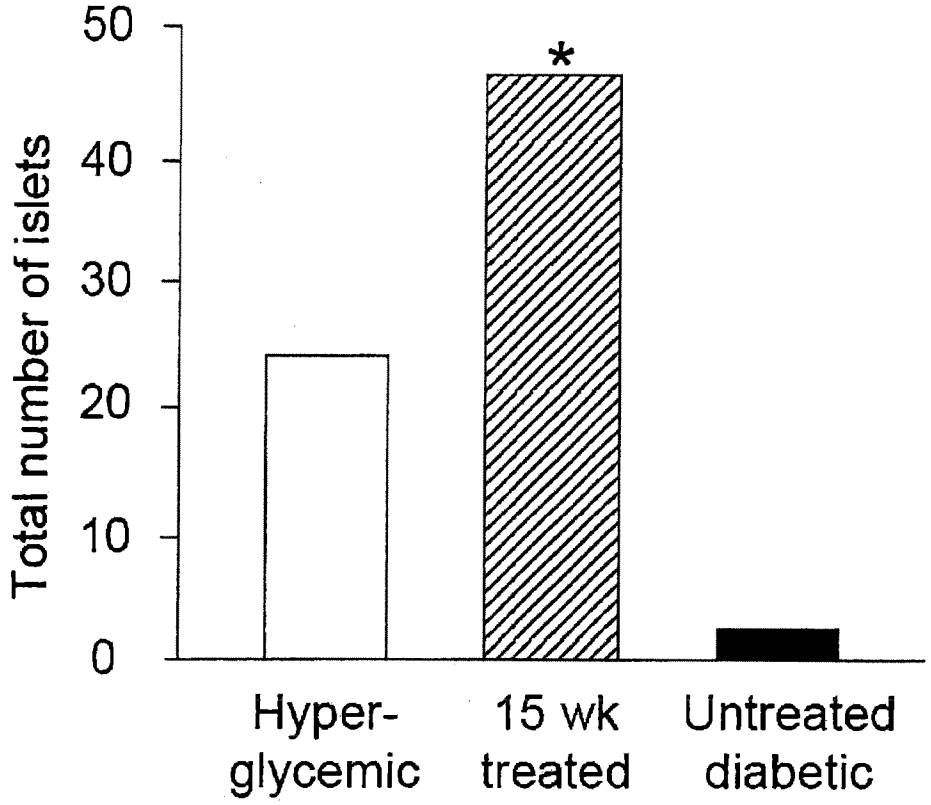

50
40
30
20
10
0
Total number of islets
*
Hyper-
glycemic
15 wk
treated
Untreated
diabetic

Figure 3

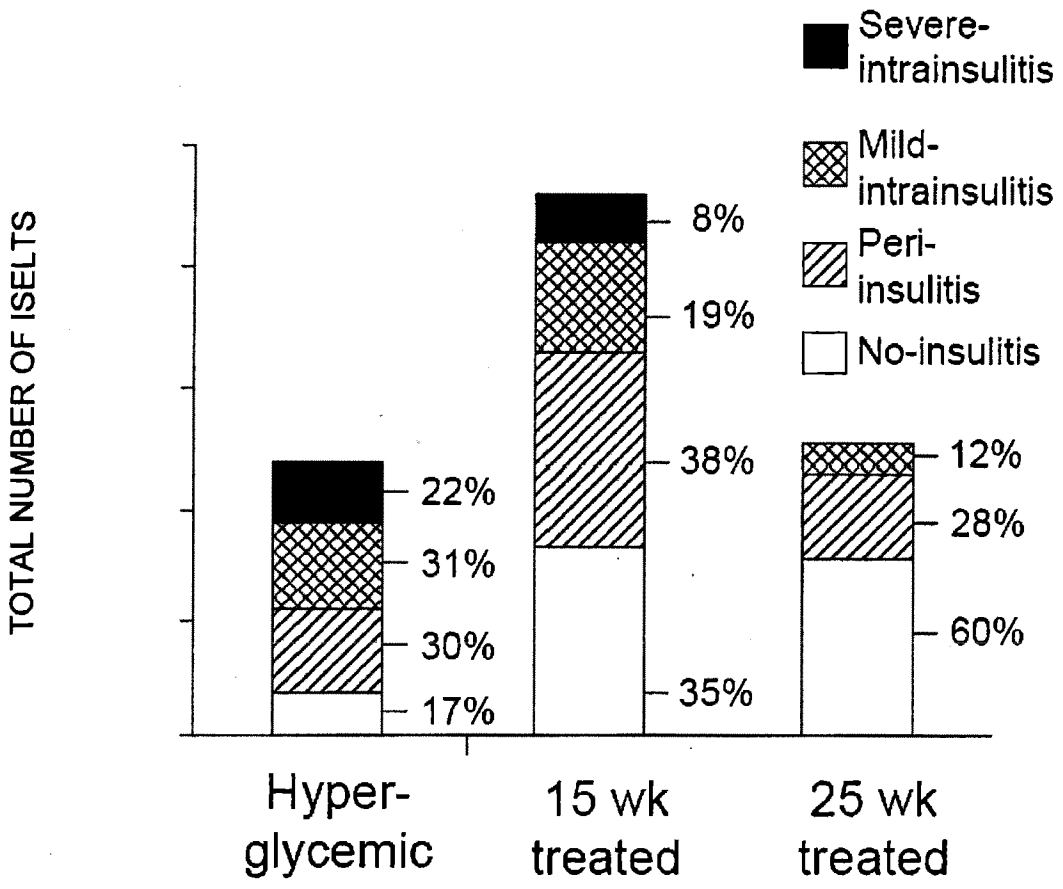

TOTAL NUMBER OF ISELTS
Severe-intrainsulitis
Mild-intrainsulitis
Peri-insulitis
No-insulitis
22%
31%
30%
17%
8%
19%
38%
35%
12%
28%
60%
Hyper-glycemic
15 wk treated
25 wk treated

Figure 4

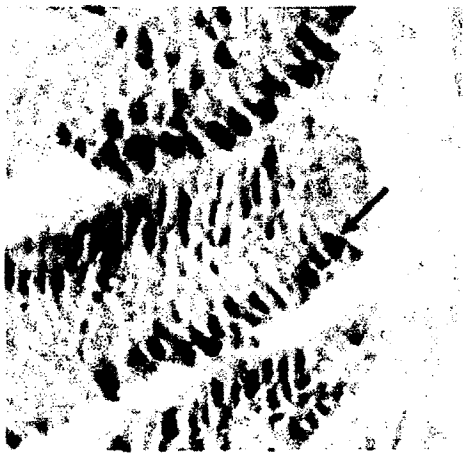


Figure 5

Normal

Figure 6

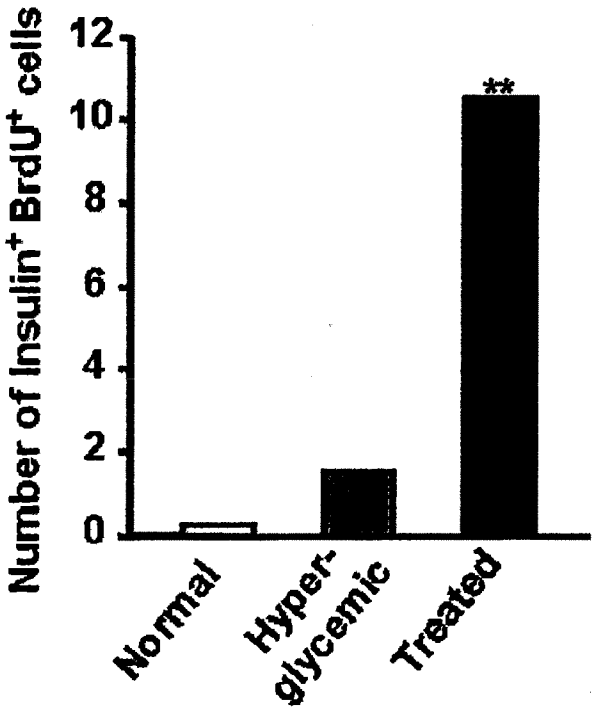
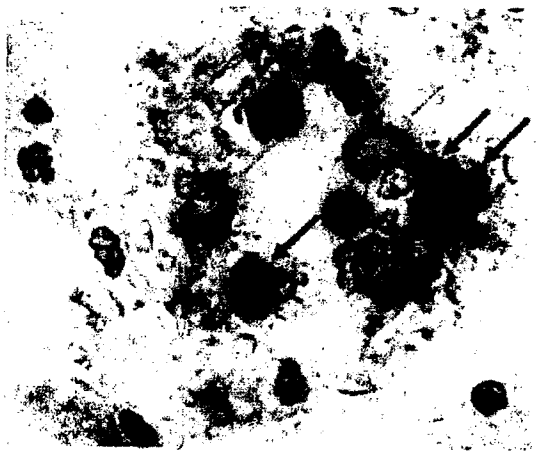

Number of Insulin+ BrdU+ cells
12
10
8
6
4
2
0
**
Normal
Hyper-glycemic
Treated

Figure 7

Figure 8



Treated

Figure 9

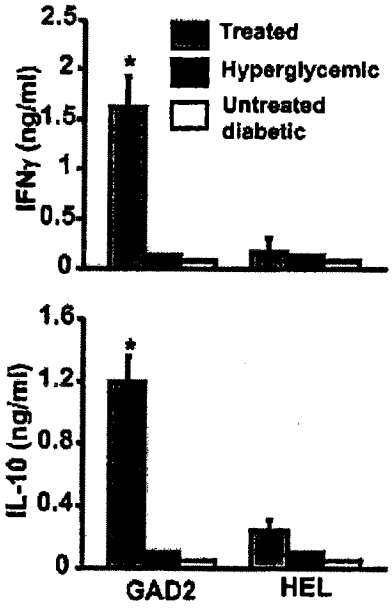

2.5
2
1.5
1
0.5
0
IFNγ (ng/ml)
Treated
Hyperglycemic
Untreated diabetic
*
1.6
1.2
0.8
0.4
0
IL-10 (ng/ml)
*
GAD2
HEL

Figure 10

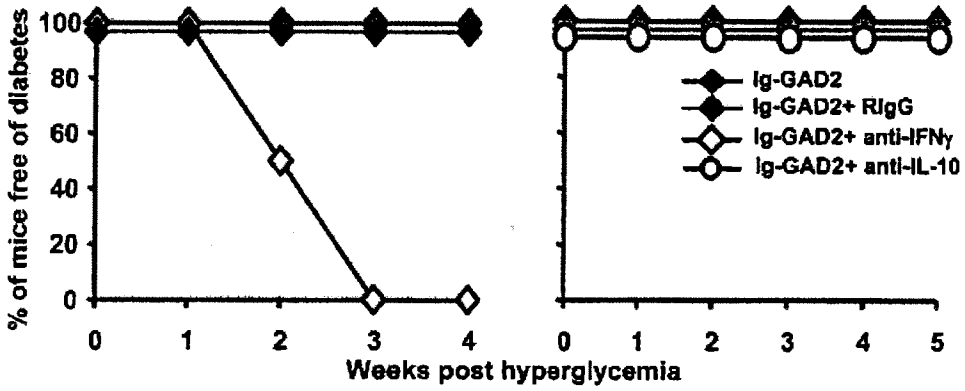

% of mice free of diabetes
100
80
60
40
20
0
0
1
2
3
4
0
1
2
3
4
5
Weeks post hyperglycemia
Ig-GAD2
Ig-GAD2+ RIgG
Ig-GAD2+ anti-IFNγ
Ig-GAD2+ anti-IL-10

Figure 11